The Prolyl-Aminodipeptidases and their Inhibitors as Therapeutic Targets for Fibrogenic Disorders

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Abstract: Many biologically active peptides are protected from general proteolytic degradation by evolutionary conserved prolines (Pro), due to conformational constraints imposed by the Pro residue. Thus the biological importance of prolyl-specific peptidases points to a high potential for drug discovery for this family of enzymes. Panels of inhibitors have been synthesized and their effects, determined in biological models, suggest the inhibition of families of enzymes with similar activities. Prolyl-specific aminodipeptidases include dipeptidyl-aminodipeptidase IV (DPP IV)/CD26, DPP8, DPP9 and fibroblast activation protease-α (FAP-α)/seprase, able to release X-Pro dipeptides from the N-terminus of peptides. DPP IV inhibitors are in clinical use for type 2 diabetes. In this review, the expression and the potential functions of prolyl-aminodipeptidases are reviewed in diseases, and the inhibitors developed for these enzymes are discussed, with a specific focus on inhibitors able to discriminate between DPP IV and fibroblast activation protease-α (FAPα)/seprase as potential leads for the treatment of fibrogenic diseases.

Key Words: Diabetes, dipeptidyl-aminodipeptidase IV, fibroblast activation protease-α, fibrogenic disorders, specific inhibition.

INTRODUCTION

Proteolytic post-translational modification of proteins and peptides is a major regulatory event for biologically active peptides. Therefore, proteolytic activities are targets for the development of drugs aiming at the treatment of human disorders, and selective inhibitors for these activities are in clinical use or are under development. Many biologically active peptides are protected from general proteolytic degradation by evolutionary conserved prolines, due to the conformational constraints imposed by the Pro residue to the peptide chain, pointing to the biological importance of prolyl-specific peptidases. Of the ~400 known human proteases only a few prolyl-specific proteases have been described, which include serine-proteases such as the cell plasma-membrane anchored amino-dipeptidyl peptidase IV (DPP IV/CD26) and fibroblast activation protease-α (FAP-α/seprase) and the intracellular DPP8, DPP9, or metalloproteases, mainly of the matrix metalloproteases (MMPs) family. Both DPP IV/CD26 and FAP-α/seprase [1-12] share the ability to release X-Pro dipeptides from the N-terminus of peptides, but in addition FAP-α/seprase also displays post-prolyl hydrolytic gelatinase and collagenase-like endoproteolytic activities (Fig. (**1**)).

The hydrolytic step may be performed extracellularly and sequentially by plasma-membrane anchored proteases, such as DPP IV and FAP-α/seprase (Fig. (**2**)), or in cell compartments.

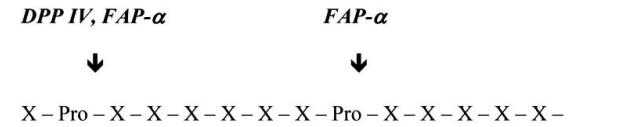

Fig. (1). Schematic representation of the hydrolytic cleavage sites of DPP IV and FAP-α/seprase.

X= any amino acid except Pro.

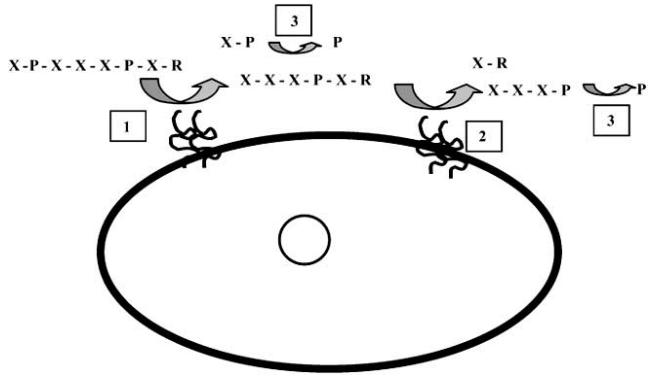

Fig. (2). Potential complementary activity of dipeptidyl peptidase-like enzymes in the processing of prolyl-substrates.

1 = DPP IV and FAP-α/seprase; 2 = FAP-α/seprase and MMPs; 3 = prolyl-specific dipeptidases and/or carboxypeptidases (not discussed in this review). X – X - X – P - R: prolyl peptide of any length and composition.

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Both proteases share common hydrolytic mechanisms and kinetics characteristics [13], and presently most developed inhibitors for these enzymes are not selective enough to differentiate their activities. Therefore enzymatic activities attributed to DPP IV in biological samples may be in fact due to FAP-α/seprase, and vice-versa. This literature survey will discuss the potential role of both enzymes in disease.

THE PROLYL-AMINODIPEPTIDASES DPP IV AND FAP-α

Dipeptidyl peptidase IV /DPP IV/CD26 (EC 3.4.14.5.) is a homodimeric type II integral membrane glycoprotein able to release X-Pro or X-Ala dipeptides from the free N-terminal sequence of peptides. DPP IV is widely expressed in epithelial and non-epithelial tissues, and is also a signaling molecule in T-cells, and an adenosine deaminase (ADA) and extracellular matrix protein binding molecule, independently of its enzymatic activity. Nearly all peptides bearing an X-Pro- or X-Ala-sequence (X is indifferent, except Pro) are potential substrates for DPP IV, including cytokines, chemokines and growth factors. DPP IV-mediated limited proteolysis of these peptides results either in their activation, modulation or initiation of degradation [7,14]. Thus endogenous inhibitors may extend the half-life of the non-hydrolyzed form of these peptides, modulate their bioactivity or block their activation. DPP IV has a dual function i) as a regulatory protease, involved in the digestion of Pro-containing oligopeptides, nutrients, and adsorption of their fragments, ii) as a binding protein, in particular for collagens I, III *via* DPP IV Cys-rich domain, and for ADA, independently of its enzymatic activity [reviewed in 15]. Inhibitors of DPP IV activity are in clinical use for type 2 diabetes [9, 14, 16-20].

Fibroblast activation protein-α, FAP-α/seprase [10] is a dimeric type II integral membrane peptidase with a molecular weight and an enzymatic activity comparable to DPP IV, but in addition seprase also displays gelatinase and collagenase activities. FAP-α/seprase is a cell surface antigen of reactive fibroblasts in cancer able of post-Pro hydrolytic activity either removing a X-Pro-dipeptide from the N-terminus of biological peptides or in the core of the peptide chain, in melanoma or sarcoma cells [21-28]. FAP-α/seprase is found at remodeling stroma in tumors and healing wounds [29]. FAP-α/seprase has been involved in tumor progression [25,30-34], liver disorders [34-37] and endothelial cell migration [38], but it is not found in normal tissues. FAP-α activity has pro-fibrogenic functions and is a regulator of cell apoptosis, adhesion and migration, independently of its enzymatic activity [36-38].

DPP IV/CD26 AND FAP-α/SEPRASE IN DISEASE

Diabetes and Metabolic Disorders

DPP IV catalyzes the cleavage of glucagon-like peptide 1 (GLP-1) from GLP-1(7-36) to GLP-1(9-36). The former is a mediator of glucose-stimulated insulin secretion, while the truncated version is an antagonist of GLP-1 for its receptor. Thus DPP IV inhibitors (DPP728, LAF237, MK0431) have therapeutic potential in long-term chronic treatment of type 2 diabetes ([39-42] and for a more detailed review, see [17]), able to dose-dependently inhibit DPP IV activity and decrease glucose in diabetic patients, with few side-effects [18, 43].

Cancer

DPP IV and FAP-α expression and/or activity are inversely correlated with the progression of carcinoma and melanoma [28, 44-50]. DPP IV was shown to be down-regulated during the neoplasic transformation of melanocytes, coincident with an increase in their invasive potential [46] and growth factor independence [51-54]. DPP IV expression and activity is anti-progressive in cancers by increasing E-cadherin expression, cell clustering [52-54] and by regulating the expression of MMPs and/or their inhibitors in carcinoma cells [52-54, 55]. However, DPP IV expression as a receptor for tumor-associated fibronectin on endothelial cells has been shown to favor tumor cell adhesion and metastasis, independently of its enzymatic activity. Thus, DPP IV may also favor cancer progression due to its adhesive properties [56,57]. No formal demonstration of the efficacy of inhibiting DPP IV in cancer has been provided. A small boronic inhibitor of DPP IV-like peptidases had anti-tumor effects, which was hypothesized to be mediated by the immune system and FAP-α inhibition [58]. DPP IV/CD26 expression and activity was lost during cancer development in lung epithelial cells, correlated with increased FAP-α expression [48,49]. FAP-α has also been involved in cancer progression [21, 22, 25, 32-34]. FAP-α is expressed by reactive fibroblasts in cancer and by melanoma or sarcoma cells [23-28, 33, 34, 59]. Published information has mostly shown that DPP IV inhibition favors progression, suggesting that inhibition of this activity may possibly be detrimental, FAP-α associated to fibroblasts and stromal cells of cancers is a likely better candidate, if FAP-α selective inhibitors can be devised. A preliminary study performed with an anti-FAP-α antibody demonstrated safety [27].

Immune Disorders

DPP IV-like activity is also important in modulating the functions of immune peptides. Many biologically active immune peptides, cytokines and chemokines, bear evolutionary conserved X-Pro-sequences that play a major role in regulating the biological functions and modulating their activity. X-Pro- truncation of chemokines and cytokines has important effects on the functions of these peptides. DPP IV-like peptidases and their inhibitors may lead to both quantitative and qualitative changes in the signaling potential of bioactive peptides (reviewed in [15], and [59-62]). CD26/DPP IV is a differentiation marker playing an integral role in the proliferation, activation and differentiation of immune cells, in particular mononuclear cells, independent of its enzyme function.

Fibrogenic Diseases

The amino acid selectivity for Pro of prolyl-specific proteases suggests that extracellular matrix (ECM) proteins [63], in particular collagens and collagen fragments may also be substrates for these enzymes, in addition to biologically active small peptides. The quality of connective tissue, especially collagens, plays important functions in the maintenance of tissue homeostasis. Fibrosis is a leading cause of

morbidity and mortality [64]. It may affect many organs, including the liver (such as liver cirrhosis), the lungs (such as idiopathic lung fibrosis and transplant-associated disorders), the kidney (such as immune disorders), but is also a response of tissue to cancer or to the implantation of therapeutic devices. Fibrosis is an improperly regulated wound healing response to tissue injury. Inflammation and fibrosis are two interrelated processes with overlapping mechanisms, involving 3 cell types: macrophages, T-lymphocytes and myofibroblasts. Following tissue injury, an inflammatory stimulus is frequently necessary to initiate tissue repair, where cytokines released by resident and infiltrating leukocytes stimulate proliferation and activation of fibroblasts to myofibroblasts. In normal situation, this phenomenon is under control, and (myo)fibroblastic response decreases and the cells disappear. When tissue repair stimulates an inappropriate pro-fibrotic response, myofibroblasts amplify the pro-fibrotic response.

Macrophages are fundamental for wound resolution, but also to fibrosis development. These phagocytes secrete factors which are chemotactic and mitogenic for endothelial cells and fibroblasts, which migrate to the site of the wound and become activated as myofibroblasts. Myofibroblasts can be derived from local resident fibroblasts, but also be recruited as fibrocytes from the bone marrow, or derived from specialized perivascular cells, the pericytes, or even from local epithelial cells by a process named epithelial–mesenchymal transition (EMT) mediated by TGF-β [65]. The increased number of (myo)fibroblasts may thus originate from an excessive proliferation or an acquired resistance to physiological apoptosis or be the consequence of EMT. Then activated myofibroblasts produce and deposit large quantities of ECM proteins, predominantly types I and III collagens, increasing tensile strength of the wound, contracting the wound and reducing its size. During this remodeling process which involves rapid synthesis and degradation of ECM proteins, synthesis overcomes degradation, which results in scar formation. In normal wound healing, scar resolution is the final process, in which reduced collagen synthesis and increased degradation, regeneration of the epithelial layer and the vascular network, and disappearance of myofobroblasts by apoptosis is observed. Therefore the modification of the proteolytic balance during the time-course of repair process is of fundamental importance. In this process MMPs secreted by myofibroblasts, and their inhibitors are important. Any disruption of this equilibrium of the proteolytic balance may result in excessive deposition of ECM, destruction of normal tissue architecture and compromised tissue function, a process which is called fibrosis.

Tissue fibrosis associated to cancer is a general problem encountered in human cancer, whose mechanisms of development are not well understood, but which results in promoting cancer progression and dysfunction of the host tissue [66]. From the known functions of myofibroblasts, it can be inferred that these cells play a major role in the onset and development of these problems. This tumoral fibrosis has been compared to a wound that does not heal. FAP-α/seprase may also be involved in cancer-dependent fibrosis development and cancer progression by aberrantly processing tissue-derived biologically-active peptides and ECM proteins. Fibrotic reaction to implanted artificial material is also a general problem encountered in tissue engineering. The mechanisms of its development are not well understood and mostly result in dysfunction of the implant and the tissue. The pathogenesis of implant fibrosis involves an initial and probably repetitive tissue injury that leads to abnormal tissue repair involving mesenchymal cells, which eventually leads to thickening of the surrounding tissue and complete obliteration of the implant and an accumulation of proliferating fibroblasts and myofibroblasts, the hallmark of tissue fibrosis.

Therefore myofibroblasts are present in low number in normal tissues, and in increased number in healing wounds, in fibrocontractile diseases, in tissue reaction to implants and in cancer. The main feature of myofibroblasts is represented by an important contractile apparatus comparable to smooth muscle cells, in particular the expression of α-smooth muscle actin (α-SMA), the production of the ECM, including the collagens, and the production of proteolytic enzymes including MMPs and FAP-α/seprase, able to degrade ECM, promote cell activation and cell migration. Importantly, collagen synthesis by myofibroblasts is strictly dependent on the availability of free proline. Therefore this amino acid is a profibrotic mediator and the enzymes able to release this amino acid may be of interest as therapeutic targets. The *de novo* expression of specific proteins able to mediate fibrosis by myofibroblasts is accepted, although the precise molecular mechanisms are not yet elucidated. Several peptide families in addition to cytokines and chemokines, have been involved in fibrotic proliferation [67-72] which may also be substrates for FAP-α/seprase. The production of MMPs by activated myofibroblasts is accepted and may represent targets for fibrosis therapy. However, activated fibroblasts also express, under a very poorly understood mechanism of regulation, FAP-α/seprase, which may represent a therapeutic target other than MMPs. Thus myofibroblasts are believed to be the main culprit in the process of fibrosis, the pathogenic deposition of the ECM, and the contraction of ECM matrix. Profibrotic cytokines include TGF-β, PDGF, IL-13, IL-4, and also fragments of ECM proteins, such as fibronectin. Myofibroblasts secrete collagens I, III IV, fibronectin, laminin and proteoglycans, and the expression of MMP-2, MMP-3 and MMP-9 is increased. Myofibroblasts also express the mannose receptor, the toll-like receptors TLR-2, TLR-4 and CD14 [73], like macrophages, and are stimulated by LPS. For all these reasons, activated myofibroblasts have become prime targets for therapeutic intervention in the process of fibrosis.

In conclusion, DPP IV and FAP-α peptidases may lead to both quantitative and qualitative changes in the signaling potential of bioactive peptides. Administration of DPP IV inhibitors in clinical trials attenuates or potentiates the effects of biological peptides involved in metabolic human diseases, depending on the context of a particular disorder, or its stage of evolution. CD26/DPP IV is involved in the development and progression of immune and metabolic disorders, but the expression of CD26/DPP IV is inversely correlated with the development and the progression of cancer, while the opposite is true for FAP-α in cancer and in fibrotic diseases. However, as families of enzymes display DPP IV-like activities, the inhibitors under development for the pep-

tidases of the DPP IV-like family must be selective enough to exclude enzymes other than their actual target peptidase. These peptidases have multiple, and likely different substrates. Therefore the effects, development and evaluation of inhibitors synthesized for their inhibition must be examined from the perspective of selectivity.

INHIBITORS OF PROLYL-DIPEPTIDYL-AMINOPEPTIDASES DPP IV AND FAP-α/SEPRASE

Inhibition of DPP IV

The Gly-Trp-Ser-Tyr-Gly sequence around the active Ser and the organisation of the catalytic triad is conserved in DPP IV and FAP-α [3,9,14,74]. The 3D structure (X-ray crystal structure) and kinetic characteristics of DPP IV have been determined [4,6,12,17,37,75-78]. The crystallized porcine enzyme is a symmetric tetramer, depending on the glycosylation of the β-propeller [4]. Each subunit comprises a C-terminal α/β–hydrolase domain and a N-terminal eight-bladed β-propeller domain [4,12]. Analogy to the related protease POP suggests that the substrate accesses the buried active site through the β-propeller, the products leaving through a different exit site. A large cavity located between the domains contains the inhibitor binding pocket (Glu^{205}, Glu^{206} and Arg^{125} residues) provided by both subunits. The Pro binding pocket is formed by a group of hydrophobic amino acids from the α/β hydrolase domain. Crystallographic studies with substrates and inhibitors bound to DPP IV have evidenced the key interactions for binding [6,79]. A positively charged N-terminus is required for binding to the negatively charged carboxylate side chains of Glu^{205} / Glu^{206} and to the hydroxyl group of Tyr^{662} (Fig. (**3**)). The residue Asn^{710} binds to the substrate/inhibitor's P2 carbonyl oxygen and the amino acid Arg^{125} positions Glu^{205} for substrate binding. This residue may also directly bind to the substrate/ inhibitors P2 carbonyl oxygen after slight rotation.

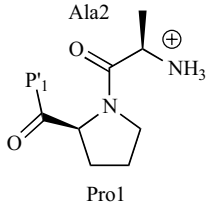


Fig. (3). DPP IV substrate (dipeptidyl peptidase activity).

Based on these structural features, several families of inhibitors have been designed, synthesized and evaluated on purified enzymes [80-82]. Most of the efforts have been devoted to the development of new therapeutic agent against type 2 diabetes.

The first class of DPP IV inhibitors comprises reversible substrate analogs with a strong emphasis on pyrrolidine derivatives. The main criterion to ensure strong inhibitory activity is the presence of a protonated amino group which also allows selectivity over prolyl-endopeptidases (Fig. (**4**)).

Early biological validation for the use of DPP IV inhibitors as tools to treat diabetes was provided by Val-Pyr (**1**) which was able to reduce plasma DPP IV activity in rats and could be used in combination with metformin to improve

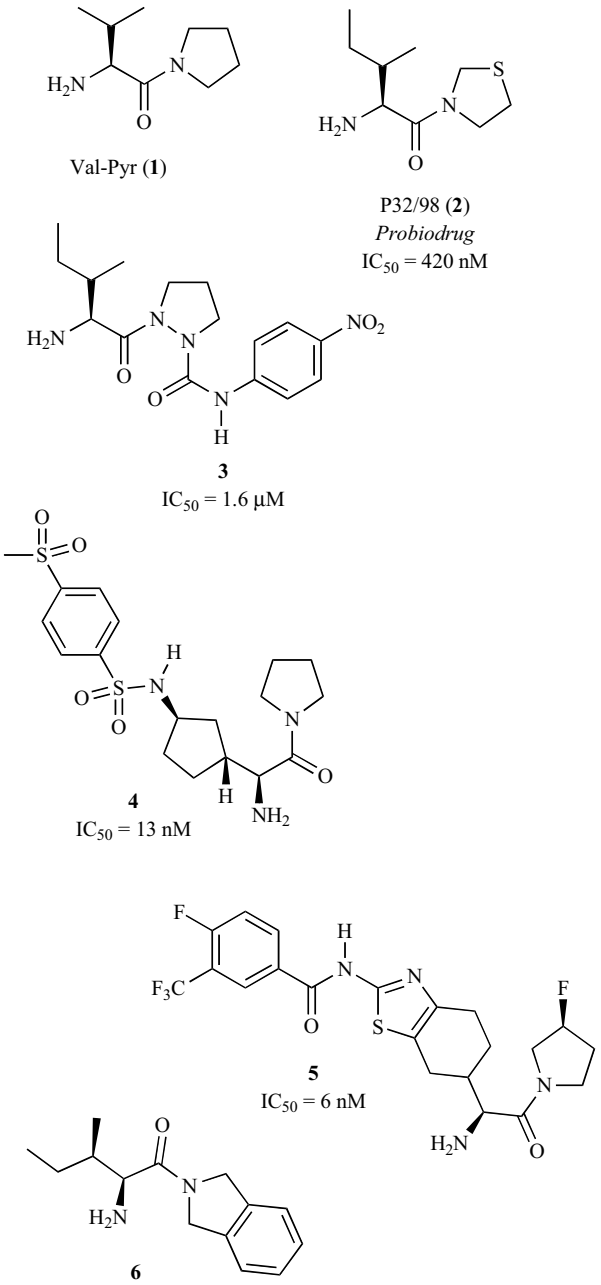


Fig. (4). Reversible DPP IV inhibitors.

glucose control [83]. Crystal structure of human DPP IV in complex with **1** gave more insight into substrate specificity [12]. In particular, location of the pyrrolidine moiety in a small hydrophobic pocket next to the active serine explains the restriction of possible residues at P1 to amino acids with small side chains (Pro, Ala, Gly). The observation that the Val side chain points into the large cavity, with no specific contacts with DPP IV, supports the fact that no specific requirements are needed for the N-terminal amino acid in the P2 position. Moreover Rasmussen *et al.* suggested that the entrance of the cavity is *via* a large opening located in the side between the hydrolase and propeller domains thus giving to the peptide the correct orientation for cleavage. After cleavage of the peptide, product may exit through a funnel

located in the center of the propeller domain. These structural indications encouraged the development of pyrrolidine-based inhibitors. Replacement of the pyrrolidine ring by thiazoline or pyrazoline motifs was also studied. Representative examples are P32/98 (**2**), developed by Probiodrug [84], and derivative **3**. P32/98 was the first DPP IV inhibitor tested in humans and displayed modest potency toward human DPP IV (IC_{50} = 420 nM) as well as low selectivity against the closely related enzymes DPP8 and DPP9. The introduction of bulky substitutents on the glycine backbone has largely improved the inhibitory properties. For instance, cycloalkyl glycine pyrrolidines demonstrated interesting inhibitory properties against DPP IV, with the cyclopentyl group affording the greatest potency (**4**) [85]. Mono-fluorination of the pyrrolidine ring led to a family of potent DPP IV inhibitors such as **5** [86]. Substitution of the pyrrolidine by a phenyl ring changed the selectivity toward DPP8/DPP9 enzymes and interestingly derivative **6** resulted in multi-organ pathology and mortality in rats, thus underlying the crucial importance of developing specific inhibitors of DPP IV if pharmaceutical use is pursued.

Another approach took advantage of the active site serine residue (Ser^{630}) to develop a family of irreversible inhibitors by covalent linkage of the serine hydroxyl group to functionalities installed on the pyrrolidine ring (Fig. (**5**)). A large panel of cyanopyrrolidines has thus been designed and synthesized, the inhibitory activity resulting from irreversible imidate formation with the active site serine residue.

The first generation of such inhibitors is exemplified by pyrrolidines **7**-**10** (Fig. (**6**)).

The cyano group ensures a very strong affinity toward DPP IV. Nevertheless, these structures have the tendency to undergo an inactivating internal cyclisation by attack of the nucleophilic glycine amino group on the electrophilic nitrile (Fig. (**7**)).

Solution stability studies have been performed and showed a half-life of 5 hours for compound **7**. Increasing the steric hindrance on the amino acid and on the pyrrolidine ring resulted in a very efficient chemical stabilization of cyanopyrrolidines as demonstrated by half life of 27 and 42 hours for **9** and **10**, respectively. Interestingly, Magnin *et al.* studied the gain in conformational stability due to both the bulk of the amino acid side chain and the introduction of a methano linker on the five-membered ring [87]. Based on the flattening effect of the five membered ring induced by the introduction of a 4,5-methano moiety on proline, the authors explored the substitution of **9** and analogues by methano linkers. Computational analysis indicated that the undesired cyclization reaction is initiated from an *anti* conformation of the amide where the amino group and the reactive cyano substituent are close to each other (Fig. (**8**)). *Ab initio* calculations demonstrated that the introduction of a *cis*-4,5-methano linker results in an increase of 0.6 kcal/mol of the energy barrier required to adopt the *trans* conformation from the ground state *syn* conformation. Moreover the energy required to assume the *anti* conformation also increases with the bulk of the amino acid side chain. This study thus explains the remarkable improvement in solution stability observed with derivative **10**. This concept led to highly potent inhibitors that entered clinical trials [82,88-90]. In particular, the adamantyl substituted Saxagliptin, developed by Bristol Myers Squibb is exceptionally potent against human DPP IV, with subnanomolar activity while introduction of aromatic substituents such as in Denagliptin resulted in a high chemical stability at 37°C (more than 250 hours). The use of a fluoroolefin amide isostere (compound **11**) led to a drastic decrease of the inhibitory activity. Substitution of the amino group of the glycine residue also decreases the rate of internal cyclization. For instance, introduction of an adamantyl group such as in Vildagliptin provided a potent DPP IV inhibitor with excellent pharmacokinetic profile and improved stability compared to the aromatic analog **15**.

The cyanopyrrolidine inhibitors described above have modest selectivity profiles against the critical isozymes DPP8 and DPP9 (< 100 fold selective). Interestingly, the ethynyl substituted derivative **17**, developed by Abbott, presented an exceptional selectivity with factors (ratios of IC_{50}) exceeding 30000 against DPP7, DPP8 and DPP9. Replacement of the cyano substituent by an electrophilic carbonyl group (compound **18**) delivered reversible DPP IV inhibitors that can form a tetrahedral intermediate with the active site serine. Nevertheless, internal cyclization followed by oxidation, rapidly gives dihydroketopyrazines such as **19**. Other modi-

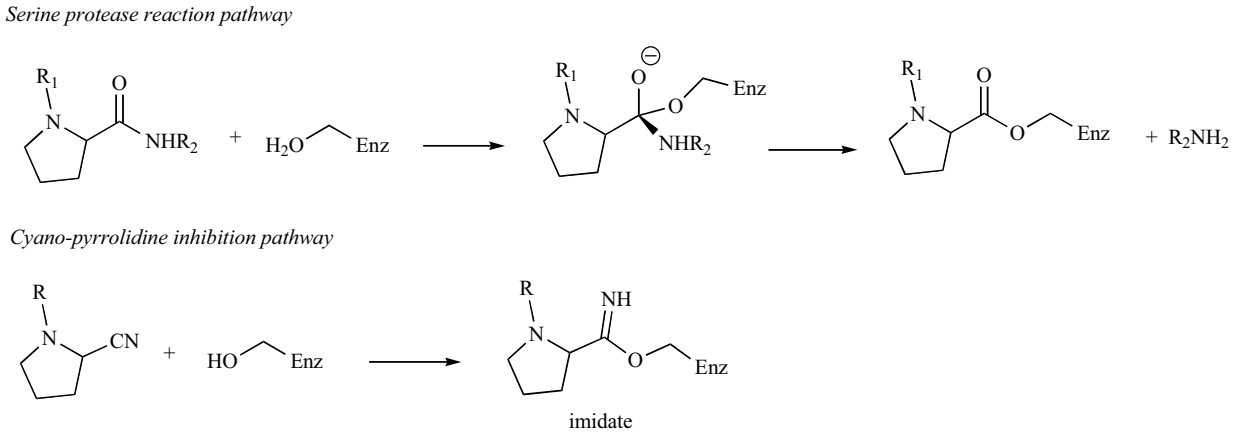


Fig. (5). Irreversible inhibition of DPP IV.

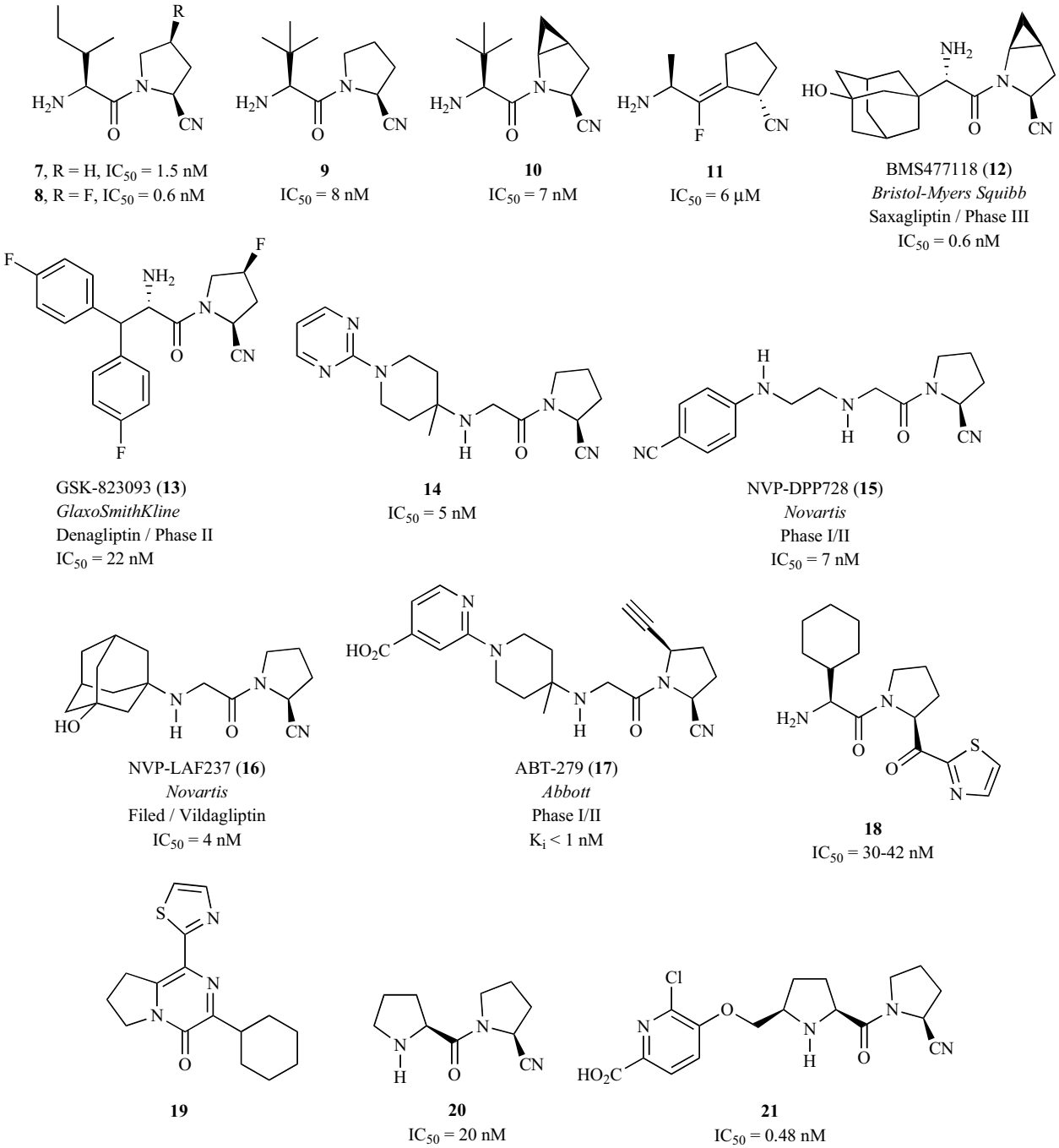


Fig. (6). Irreversible DPP IV inhibitors.

fications, such as introduction of a second pyrrolidine unit at the P2 residue led to a promising family of potent DPP IV inhibitors [91]. While the unsubstituted congener **20** showed very low stability (half life of 1.3 hour), aromatic ethers such as in **21** resulted in strong DPP IV inhibition (subnanomolar activity), increased stability and selectivity factors higher than 1000 toward DPP7, DPP8, DPP9 and FAP-α.

A third class of DPP IV inhibitors comprises reversible non peptidic heterocyclic derivatives (Fig. (**9**)).

Aminomethyl pyridine and pyrimidine derivatives have been reported as potent inhibitors of DPP IV, such as compound **22** which exhibited a 100 picomolar activity. The binding motif of such inhibitor is based on the filling of the P1 pocket by the dichlorophenyl group, cation-π interaction

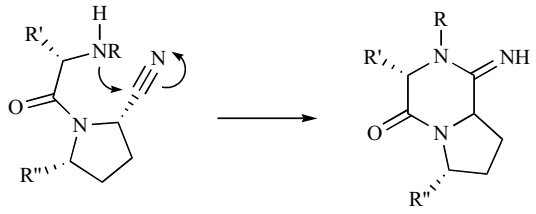


Fig. (7). Inactivation of the inhibitors through cyclization.

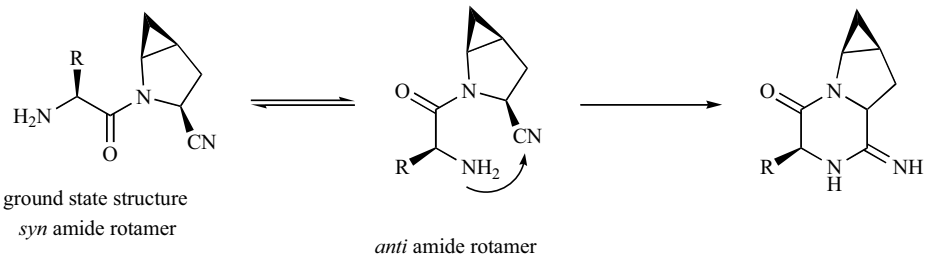


Fig. (8). Conformational stability of cyano substituted DPP IV inhibitors.

between the pyrimidine moiety and Arg^{125} and interactions of the aminomethyl substituent with Glu and Tyr residues in the active site. Xanthine based derivatives also demonstrated potent inhibitory activity on DPP IV as illustrated by BI 1356 which benefits from a long duration of action. This long lasting effect, combined with potent and selective inhibition of DPP IV (selectivity factors of at least 10000 related to DPP8 and DPP9) confers to **24** the potential to become the first truly once-a-day agent for the treatment of type 2 diabetes [92]. Simplification of the xanthine core structure to an uracyl moiety was investigated by Takeda who came up with the potent DPP IV inhibitor Alogliptin. Recently submitted to FDA approval for treatment of type 2 diabetes [93], this compound presents selectivity factors of 20000 toward DPP8 and DPP9. Merck has explored the incorporation of a β-amino acid moiety and developed Sitagliptin which represents a novel approach to inhibit DPP IV. Recently approved by the FDA for the treatment of type 2 diabetes, this derivative contains a haloaromatic moiety to bind to the P1 pocket instead of a typical protease warhead. The β-amino group interacts with both Glu residues of the active site and selectivity factors toward DPP8 and DPP9 are also excellent. Introduction of a cyclohexylamine group to rigidify the structure was proposed by Gao [94]. Based on docking studies, he developed analog **27** which contains a pyrrolopyrimidine moiety and affords higher potency against DPP IV than the parent Sitagliptin.

From the first series of DPP IV inhibitors that closely mimicked the enzyme substrate, many synthetic efforts have been devoted toward the development of extremely potent derivatives that display excellent selectivity regarding the closely related enzymes DPP8, DPP9 and FAP-α. Many pharmaceutical companies have thus proposed DPP IV inhibitors as promising new agents against type 2 diabetes. The safety and efficacy profiles of the more advanced DPP IV inhibitors are well suited for the treatment of such a chronic disease, either as a monotherapy or in association with other established therapies.

Inhibition of FAP-α

The recent solving of the crystal structure of the extracellular domain of human FAP-α allowed the investigation the substrate specificity of this enzyme [95]. FAP-α and DPP IV

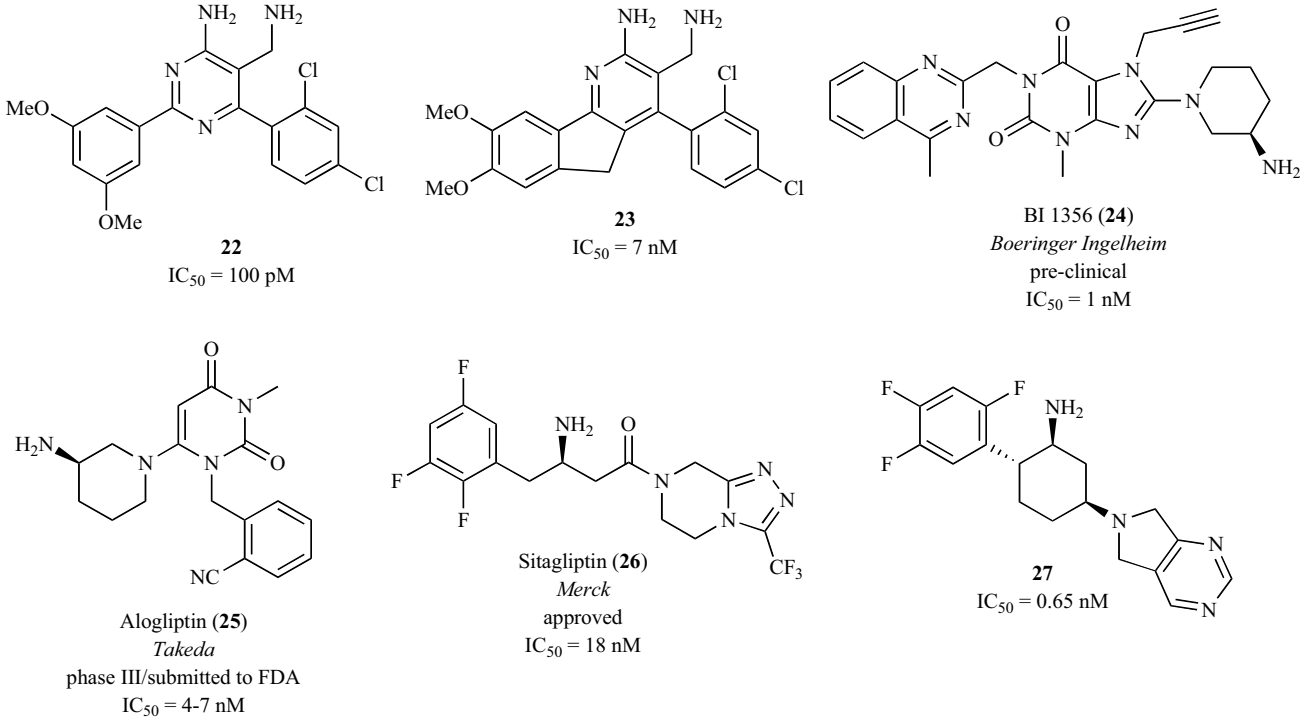


Fig. (9). Reversible non peptidic inhibitors.

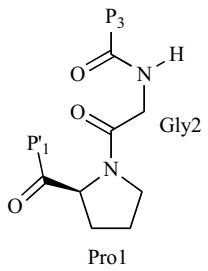
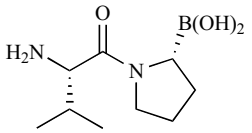


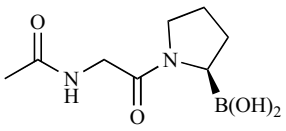
Fig. (10). Endopeptidase substrates.

have a sequence identity of 50% and FAP-α exhibits a DPP IV-like fold featuring a α/β-hydrolase domain and an eight–bladded β-propeller domain. At the interface of these two domains, the FAP-α catalytic triad is composed of residues Ser^{624}, Asp^{702} and His^{734}. As for DPP IV, dimerization of FAP-α is required for its catalytic function. The S1 specificity pocket is a well defined hydrophobic site which optimally accommodates a Pro residue while the S2 hydrophobic pocket can host large hydrophobic and aromatic residues. The main difference between the binding pockets of the two enzymes is the presence of Asp^{663} in DPP IV which corresponds to Ala^{667} in FAP-α. These residues are largely responsible for the changes in activities between both enzymes. While DPP IV and FAP-α both display exopeptidase activity with higher catalytic efficiency for DPP IV, FAP-α alone has endopeptidase activity for which endogenous substrates are still mainly unidentified. Wolf *et al.* [96,97] have determined that DPP IV Asp^{663} and FAP-α Ala^{667} regulate protease specificity with the Asp residue resulting in a more pronounced negatively charged pocket. The FAP-α specificity model predicts that beyond P1 Pro residue, the substrates must contain a small amino acid able to adopt a positive phi value in the Ramachandran plot to avoid steric clashes between the protease and the P3 residue side chain and the P3 residue carbonyl group of the substrate (Fig. (**10**)).

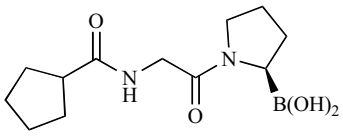
Glycine is the only amino acid that fulfills these criteria. Small D-amino acid may also replace Gly at P2. The endopeptidase activity of FAP-α is thus restricted to Gly-Pro



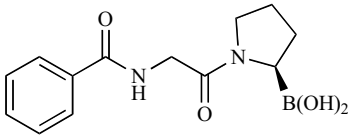
28
PT-100
Talabostat / phase I



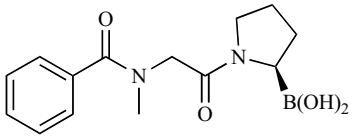
29
K_i (FAP) = 23 nM
K_i (DPPIV) = 377 nM
selectivity factor: 16.4



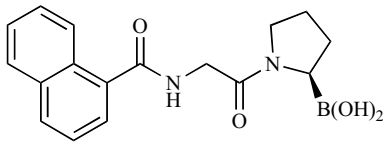
30
K_i (FAP) = 20 nM
K_i (DPPIV) = 9080 nM
selectivity factor: 454



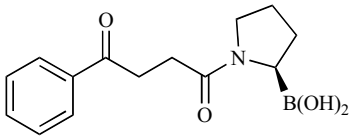
31
K_i (FAP) = 142 nM
K_i (DPPIV) = 38400 nM
selectivity factor: 270



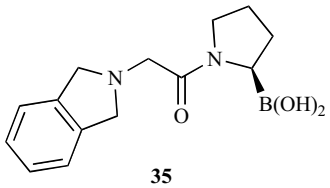
32
K_i (FAP) = 191 nM
K_i (DPPIV) = 11300 nM
selectivity factor: 59



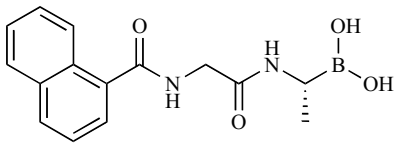
33
IC_{50} (FAP) = 1.8 nM



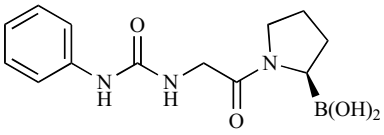
34
K_i (FAP) = 94 nM
K_i (DPPIV) = 451000 nM
selectivity factor: 4798



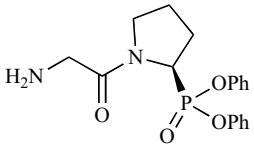
35
K_i (FAP) = 7.5 nM
K_i (DPPIV) = 22700 nM
selectivity factor: 3027



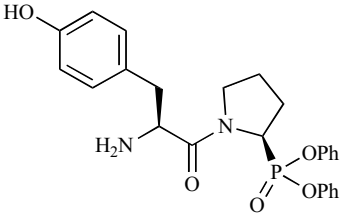
36
IC_{50} (FAP) = 8.2 μM



37
IC_{50} (FAP) = 7.1 nM



38
K_i (FAP) = 3270 nM
K_i (DPPIV) = 59 nM



39
K_i (FAP) = 2590 nM
K_i (DPPIV) = 14200 nM
selectivity factor: 5.5

Fig. (11). FAP-α inhibitors.

containing substrate while the dipeptidyl peptidase activity of DPP IV removes P_2-Pro_1 or P_2-Ala_1 dipeptides from the N-terminus of the substrate with P2 representing any amino acid. Further studies were devoted to the understanding of FAP-endopeptidase activity mechanisms [98]. The Ala^{657} and five conserved active site residues (Arg^{123}, Glu^{203}, Glu^{204}, Tyr^{656}, Asn^{704}) promote FAP-α endopeptidase activity *via* distinct mecanisms of transition state stabilization. Conserved active site residues bind both dipeptidyl peptidase and endopeptidase substrates and orient the scissile bond for cleavage. Nevertheless, Ala^{657} does not bind substrates but regulates protease specificity by controlling the geometry of the active site. In FAP-α, the Glu^{203} residue has enough conformational freedom to allow endopeptidase activity. In DPP IV, Glu^{206} is bound by Asp^{663} residue which reduces its conformational freedom. This constraint prevents endopeptidase activity.

Recently the amino boronic dipeptide Val-BoroPro (**28**, PT-100) [99], an inhibitor of the dipeptidyl peptidase activity of DPP IV and FAP-α, demonstrated hematopoietic stimulation and anti-tumor effects in preclinical trials. Nevertheless, DPP IV is not involved in this antitumor activity, thus suggesting that the molecular target of PT-100 is FAP-α (Fig. (**11**)).

Based on their study on the endopeptidase activity of FAP-α, Wolff and coll. reported N-acyl-Gly-BoroPro (**29**) as a specific inhibitor of FAP-α with selectivity factors (measured as ratios of Ki values) ranging from 16 to 5400 related to DPP IV, DPP7, DPP8 and DPP9 while the non acylated analog inhibited both DPP IV and FAP-α. Further optimization of the inhibitory activity against FAP-α led to the preparation of a family of boronic peptides which presented high and selective affinity for FAP-α [100]. In particular, substitution of the terminal *N*-acyl group induced a gain in selectivity against DPP IV. For instance, the cyclopentyl- or phenyl-substituted derivatives **30** and **31** presented selectivity factors of 454 and 270, respectively. An increase in the size of the aromatic substituent improved the affinity toward FAP-α (see **33**) [101]. Interestingly, the amide NH group does not seem to be determinant for the inhibitory activity as cyclic amide **35** and ketone **34** maintained a strong inhibition of FAP-α (K_i values of 7.5 and 94 nM, respectively) as well as high selectivity factors related to DPP IV. Urea type substituents also provided strong inhibitory activity against FAP-α as illustrated by **37**. Nevertheless, whereas DPP IV can be inhibited by an extremely large panel of peptidic and non-peptidic structures, FAP-α requires the Pro moiety for recognition. The non prolinic derivative **36** showed a dramatic loss of activity toward FAP-α. Replacement of the boronic ester moiety by a phosphonate group was also envisaged [102]. While the Gly-Pro derivative **38** displayed higher affinity for DPP IV than for FAP-α, the Tyr-Pro analogue **39** showed a modest selectivity factor in favor of FAP-α.

CONCLUSION

As DPP IV has been recognized since several years as a useful target in the treatment of type 2 diabetes, very large families of selective inhibitors of this protease have been designed and evaluated. Tremendous efforts from both academic laboratories and pharmaceutical companies have led to a new generation of anti-diabetic agents. The endopeptidase activity of FAP-α has been evidenced only very recently so that few specific inhibitors have been reported. Nevertheless, the narrow endopeptidase specificity of FAP-α, restricted to Gly-Pro substrates, has been exploited to design efficient inhibitors. Inhibiting FAP-α protease activity or disrupting of the signaling of FAP-α complexes with other surface molecules are appealing approaches to slow tumor progression and fibrosis development. FAP-α can be considered as a therapeutical target for the treatment of liver fibrosis [103] as this protease is only expressed on activated hepatic stellate cells, not in normal adult liver. FAP-α is also dominantly expressed in areas of tissue remodeling, which are regions with active fibrogenesis. In order to go further in the development of therapeutic approaches based on FAP-α inhibitors for the treatment of cancer and liver fibrosis, the natural substrates of FAP-α still need to be identified.

ABBREVIATIONS

ADA	=	Adenosine deaminase
Ala	=	Alanine
Arg	=	Arginine
Asn	=	Asparagine
Asp	=	Aspartic acid
Cys	=	Cysteine
DPP IV	=	Dipeptidyl-aminodipeptidase IV
DPP7	=	Dipeptidyl-aminodipeptidase 7
DPP8	=	Dipeptidyl-aminodipeptidase 8
DPP9	=	Dipeptidyl-aminodipeptidase 9
ECM	=	Extracellular matrix
EMT	=	Epithelial-mesenchymal transition
FAP-α	=	Fibroblast activation protease-α
GLP-1	=	Glucagon-like peptide 1
Glu	=	Glutamate
Gly	=	Glycine
His	=	Histidine
LPS	=	Lipopolysaccharide
MMPs	=	Matrix metalloproteases
POP	=	Prolyl oligopeptidase
Pro	=	Proline
Ser	=	Serine
α-SMA	=	α-smooth muscle actin
TGF-β	=	Transforming growth factor beta
TLR	=	Toll-like receptor
Trp	=	Tryptophan
Tyr	=	Tyrosine
Val	=	Valine

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