On The Edge of Validation – Cancer Protease Fibroblast Activation Protein

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Abstract: Numerous studies implicate the prolyl peptidase, fibroblast activation protein (FAP) in tumorigenesis; however, FAP-selective inhibitors have not yet been developed to fully validate FAP as a therapeutic target. Herein, we review recent efforts aimed at validating and inhibiting FAP for cancer therapy and highlight future directions for successful targeting of this prolyl peptidase.

Key Words: FAP, DPP4, dipeptidyl peptidase, prolyl peptidase, inhibitor, cancer.

INTRODUCTION

Prolyl peptidases including fibroblast activation protein (FAP), dipeptidyl peptidase-4 (DPP4), DPP7, DPP8, DPP9, and prolyl oligopeptidase (POP), are serine proteases of peptidase clan SC uniquely defined by their ability to cleave peptides following Pro residues and their association with human disease [1, 2]. DPP4, the best characterized prolyl peptidase, proteolytically inactivates the insulinotropic peptides glucagon-like peptide-1 and gastric inhibitory polypeptide and inhibition of this activity is a clinically validated treatment for type II diabetes [3]. Inhibition of FAP, the prolyl peptidase most homologous to DPP4, holds therapeutic promise as FAP activity promotes tumorigenesis in preclinical models and may also contribute to other diseases associated with activated stroma including rheumatoid arthritis, osteoarthritis, cirrhosis, and pulmonary fibrosis. However, FAP-selective inhibitors have not yet been developed to fully validate FAP as a therapeutic target. Herein, we review recent efforts aimed at validating and inhibiting FAP for cancer therapy and additionally highlight future directions for successful targeting of this prolyl peptidase.

FAP DISCOVERY

Two groups independently discovered FAP and characterized the enzyme as a cancer-associated protease related to DPP4. In 1994, the first group led by Lloyd Old and Wolfgang Rettig, used expression cloning to identify FAP as the antigen recognized by monoclonal antibody F19, an antibody selective for "activated" fibroblasts of cancer stroma [4]. Subsequently, Wen-Tien Chen's group isolated seprase, a melanoma protease associated with invadapodia and cellular invasiveness and found that it was identical to FAP [5]. Full-length cDNAs from both groups encoded a 760 amino acid protease highly homologous to DPP4 (48% amino acid identity) and predicted an identical domain structure, consisting of an N-terminal cytoplasmic tail, a transmembrane region, and an extracellular portion containing a β–propeller domain followed by an αβ–hydrolase domain [4, 6]. The proteases similarly shared a dimeric structure (Fig. (**1**)) and demonstrated dipeptidyl peptidase (DPP) activity against P_2-Pro-containing peptides (Fig. (**2**)). However, unlike DPP4, FAP hydrolyzed gelatin and had little normal tissue expression [7-9].

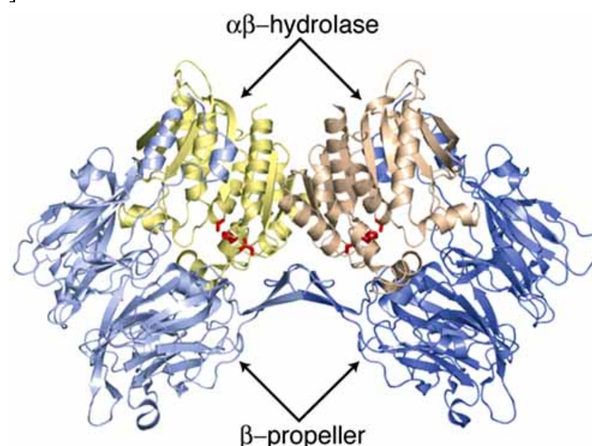

Fig. (1). Cyrstal structure of the FAP dimer (pdb 1Z68). The catalytic triad residues of each monomer are highlighted in red.

FAP IN TUMORIGENESIS

Immunohistochemistry with monoclonal antibody F19 revealed FAP's remarkable, cancer-specific expression pattern, which localized to stromal fibroblasts in all major solid tumors and to tumor cells of sarcomas [8, 9]. Gene expression profiling additionally showed high FAP RNA levels in cancer tissues and low levels in normal tissues [10]. These increases in FAP RNA and protein correlate with marked increases in FAP activity in tumors as detected by immmunocapture assays or activity-based profiling [7, 11, 12]. Notably, in colon cancer, FAP activity associates with advanced clinical stage [12] and high FAP expression in metastatic disease correlates with worse survival, suggesting FAP acts in tumor progression [13].

Preclinical studies further suggest causative roles for FAP in tumorigenesis. For example, FAP overexpression in

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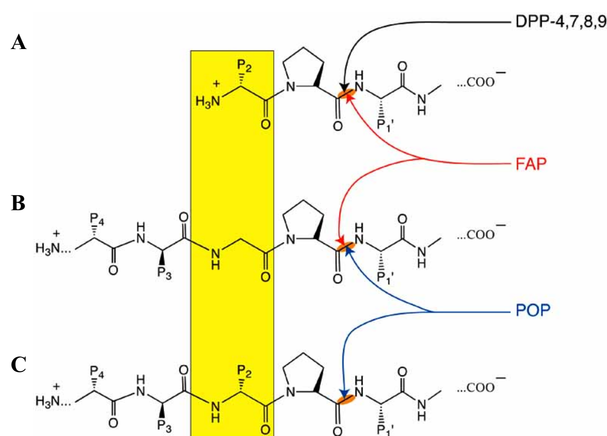

Fig. (2). Substrate specificity of prolyl peptidases. The scissile bond is highlighted in orange and differences in P_2 specificity in yellow. **A.** DPP activity requires a free N-terminus. **B.** FAP endopeptidase activity requires a Gly at P_2. **C.** POP endopeptidase activity demonstrates broad specificity at P_2.

fibroblasts or breast cancer cells enhances xenograft tumor take and growth and this correlates with increased angiogenesis in the breast cancer model [14-16]. By contrast, overexpression of catalytically inactive FAP does not promote tumor growth and inhibition of FAP activity with polyclonal α-FAP antisera attenuates tumor growth, suggesting that FAP activity enhances tumorigenesis [14, 15]. Loss of function studies with siRNAs or antisense constructs targeting FAP demonstrate that FAP enhances tumor cell survival and promotes matrix invasion *in vitro* [17-19]. These studies clearly link FAP to the cancer phenotype; however, further validation studies are required to evaluate tumorigenesis in FAP-deficient mice [20] and to delineate how FAP-dependent proteolysis contributes to oncogenic signaling.

Although most work has focused on FAP's function in cancer, marked FAP expression also occurs in other conditions associated with activated stroma, including wound healing, arthritis, and fibrosis of the lung and liver [4, 9, 21-24]. However, with the exception of liver fibrosis, a contributing role for FAP in these processes has not been demonstrated. In this case, FAP-deficient mice show reduced hepatic fibrosis and inflammation compared to wild type mice following chronic liver injury with carbon tetrachloride [25]. The mechanism underlying the reduced fibrosis is unclear; however, the data suggest that FAP may have important functions not only in cancer, but in other diseases associated with activated stroma.

To better understand FAP's role in tumorigenesis, we and others have targeted FAP with antibodies and small molecule inhibitors. Although generation of activity-blocking monoclonal antibodies has been difficult [26], studies with small molecule inhibitors appear promising. To date, however, only Val-prolineboronic acid (boroPro) (Talabostat (PT-100), **1**; Fig. **3**), a compound initially developed to inhibit DPP4 [27], has been tested extensively in tumor models [28]. Given FAP's homology to DPP4, Adams *et al*. reasoned that Val-boroPro would inhibit FAP and hypothesized that the compound might have anti-tumor activity. Supporting this, they found that Val-boroPro had potent antitumor activity against several tumors with stromal FAP expression, including sarcomas, melanomas, mastocytomas and lymphomas and that the compound remained active against tumors in DPP4 knockout mice. Subsequently, others confirmed the anti-tumor efficacy of Val-boroPro and correlated this effect with FAP inhibition [15]. However, it is now known that Val-boroPro non-selectively inhibits most prolyl peptidases (Table **1**) [29-31], making the mechanism of the inhibitor's compelling anti-tumor efficacy unclear.

Val-boroPro's preclinical efficacy prompted clinical testing in several tumor types. The inhibitor advanced to Phase III clinical trials in non-small cell lung cancer, but the FDA stopped testing as interim analyses showed that patients receiving Val-boroPro in combination with docetaxel had no clinical benefit and worsened survival relative to the placebo group[1]. It is unclear whether this result related to the non-selective nature of the inhibitor, off target effects, or other factors. However, recent studies suggest that inhibitor selectivity may be particularly important as inhibition of DPP8 and DPP9 causes toxicity and mortality in preclinical models [30].

FAP SUBSTRATE SPECIFICITY

To identify peptide-motifs for FAP-selective inhibitors, we used synthetic peptide substrate libraries to fully define FAP's specificity [32]. As early studies demonstrated that FAP cleaves certain DPP4 substrates and displays endopeptidase activity against gelatin and the proteinase inhibitor α_2-antiplasmin (α_2AP) [7, 33, 34], we synthesized coumarin-based DPP (P_2-Pro-) and endopeptidase (Ac-P_2-Pro-) substrate libraries [32]. The DPP substrate library showed that FAP, like DPP4, has little P_2 specificity, tolerating most amino acids at this position. Strikingly, however, the endopeptidase substrate library revealed a strict requirement for Gly at P_2. The requirement for Gly at P_2 was confirmed with a library of intramolecularly quenched fluorescent peptide substrates based on the FAP cleavage site in α_2AP [35]. This library also showed that d-Ala or d-Ser can replace Gly at P_2 and that FAP prefers small, uncharged amino acids at P_3, but tolerates most amino acids at P_4, P_1' and P_2'. Thus, FAP is a dual specificity protease that displays DPP and Gly-Pro-cleaving endopeptidase activities. This dual specificity distinguishes FAP from DPPs-4, -7, -8 and -9, which display only DPP activity and from POP, which has only endopeptidase activity (Fig. (**2**)) [36-39].

FAP STRUCTURE AND ENDOPEPTIDASE MECHANISM

Structural and mutagenesis studies have defined the molecular determinants of FAP's unique substrate specificity [40, 41]. The FAP crystal structure shows that both the β-propeller and αβ-hydrolase domains contribute important residues to the active site, which lies at the interface of the two domains (Fig. (**1**)) [40]. The structure was solved without bound substrate; however, the active site architecture is remarkably similar to DPP4, with complete conservation and

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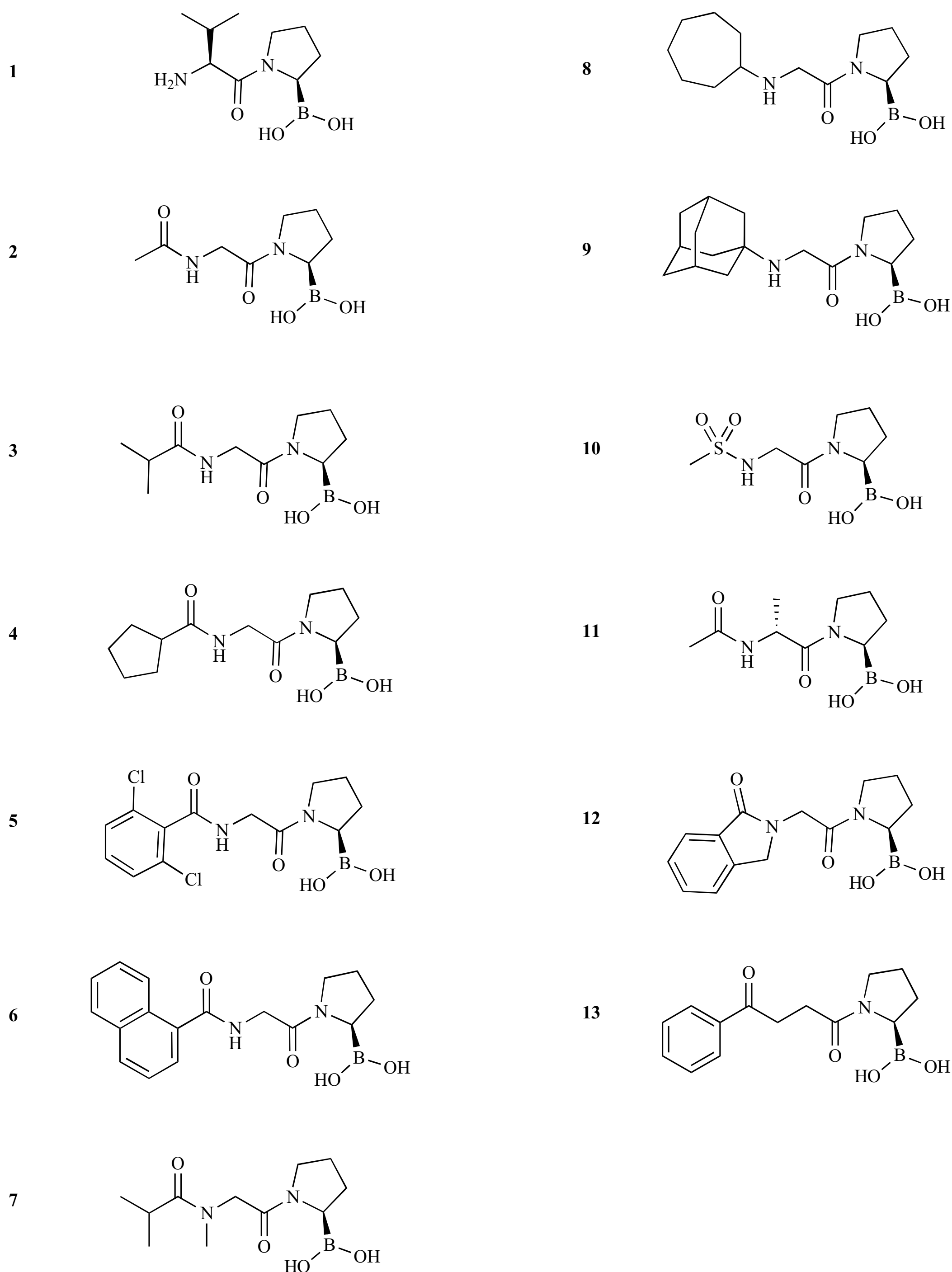

Fig. (3). BoroPro inhibitors.

similar positioning of the known DPP4 substrate-binding residues (Fig. (**4**)). At S_1, several hydrophobic residues (Tyr625, Val650, Trp653, Tyr656, Tyr660 and Val705) create a lipophilic pocket that best accommodates a Pro residue (Fig. (**5**)). At S_2, Arg123 and Asn704 bind the substrate's P_2 carbonyl oxygen and Glu203, Glu204 and Tyr656 bind the substrate's N-terminus (DPP substrate) or P_2 amide NH (endopeptidase substrate). Striking decreases in both DPP and endopeptidase activity are observed with Ala mutants of any conserved S_2 residue, confirming their critical importance for FAP activity [41].

Surface representations of the FAP active site illustrate the steric requirements for DPP and endopeptidase substrates. As observed with DPP4 substrate-binding (Fig. (**5A**)), FAP binds the DPP substrate's P_2 carbonyl and terminal amine, but does not contact the P_2 side chain, which ori-

Table 1. Inhibitory Activity (K_i, nM or (IC_{50}, nM)) of Selected BoroPros

Compound	FAP	DPP4	DPP8	DPP9	DPP7	POP	Reference
1	6.2	0.17	(4)	(11)	(310)	> 10,000[1]	[29,30,41]
2	23	377	19,100	8800	125,000	211	[32]
3	51	4300				4.5	[44]
4	20	9080				2.3	[44]
5	12	6871				4.4	[44]
6	(1.8)						[45]
7	265	19,500				13	[44]
8	(150)	(7.8)			(1.8)		[31]
9	(46)	(8)			(420)		[31]
10	246	1430				1359	[44]
11	350	>500,000				2705	[44]
12	7.5	22,700				3030	[44]
13	94	451,000				1.7	[44]

[1]Wolf, BB, unpublished data

ents away from the active site (Fig. (**5B**)). This allows broad P_2 specificity, as observed with the P_2-Pro substrate library. In contrast, FAP's S_2 site requires endopeptidase substrates with a small P_2 amino acid and a positive phi dihedral angle (Fig. (**5C**)). This requirement explains FAP's limited endopeptidase specificity as Gly is the only natural amino acid that readily adopts a positive phi dihedral angle.

Since the substrate-binding residues are completely conserved between FAP and DPP4, one or more non-substrate binding residues must account for the observed differences in protease specificity. Several non-conserved residues are located near the FAP active site, but mutagenesis studies show that only Ala657, which corresponds to DPP4 Asp663, is important for specificity (Fig. (**4**)) [40, 41]. Ala657 is critical for FAP endopeptidase activity but diminishes DPP activity relative to Asp as in DPP4. In contrast, DPP4 Asp663 potentiates DPP activity but markedly attenuates endopeptidase activity. These residues additionally dictate protease inhibition [41], with the Ala enhancing reaction with endopeptidase inhibitors and the Asp favoring inhibition by DPP inhibitors (see below). Mechanistically, the Ala and the Asp likely regulate specificity by modulating the conformation and charge of conserved residue FAP Glu204/DPP4 Glu206 (Fig. (**4**)). The Ala allows FAP Glu204 a high degree of conformational freedom, which appears necessary for endopeptidase activity. Conversely, the carboxylate of DPP4 Asp663 allows binding and deprotonation of Glu206, which conformationally restrains the Glu and strongly disfavors binding of uncharged endopeptidase substrates. Interestingly, the Glu-Asp dyad is conserved in DPP8 and DPP9, which lack endopeptidase activity, suggesting that the dyad may similarly regulate their specificity and inhibition.

FAP Inhibitors

BoroPros

As FAP's activity against Gly-Pro-based endopeptidase substrates distinguishes it from most other prolyl peptidases, we hypothesized that inhibitors containing an acyl-Gly-Pro-based motif might inhibit FAP selectively. To assess this, we synthesized and tested Ac-Gly-boroPro (**2**, Fig. (**3**)), which contained the optimal substrate motif coupled to a boronic acid electrophile capable of forming a boronate adduct with the catalytic serine [32]. We coupled the boronic acid electrophile to the substrate motif because electrophiles markedly enhance the potency of dipeptide-based DPP4 inhibitors such as pyrrolidines and thiazolidines [27, 42, 43]. Ac-Gly-boroPro inhibited FAP with a K_i of 23 nM and provided moderate selectivity (16-fold) against DPP4 and marked selectivity (380-5400-fold) against DPP7, DPP8 and DPP9 (Table **1**). Less selectivity (9-fold) was observed against POP, the only other prolyl peptidase with endopeptidase activity. Thus, the N-acyl-Gly-Pro motif allows effective inhibition of prolyl peptidases with endopeptidase activity and provides selectivity against DPPs.

We further explored the structure-activity relationship (SAR) of the N-blocking group with a series of N-acyl-Gly-boroPro inhibitors (Fig. (**3**), Table **1**) [44]. Replacing the acetyl group of Ac-Gly-boroPro with bulkier substituents including alkyl- (**3**), cycloalkyl- (**4**), and aryl-groups (**5**) generally enhanced selectivity against DPP4 and maintained potency against FAP. This likely relates differences in the hydrophobicity of the FAP and DPP4 S_3 subsites, with the more hydrophobic FAP S_3 subsite more readily accommodating hydrophobic N-blocking groups (Fig. (**6**)). In contrast

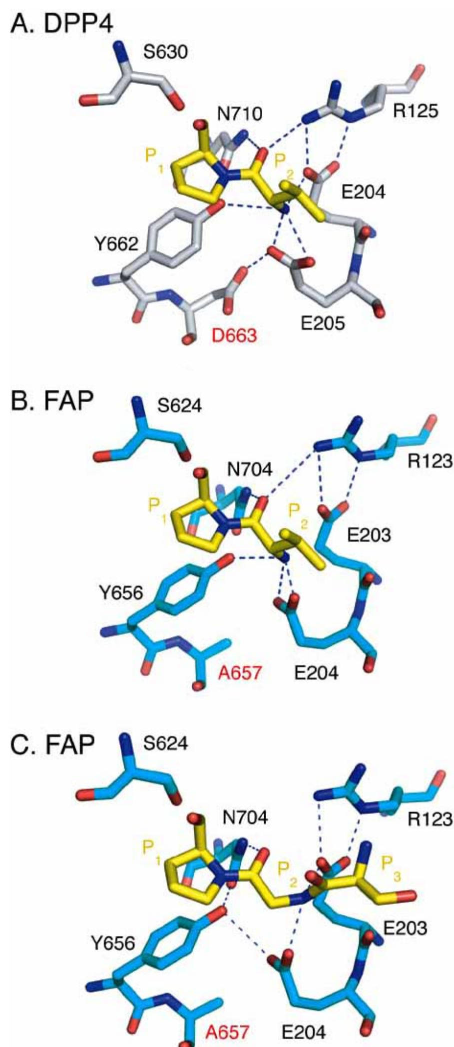

Fig. (4). DPP4 and FAP active sites. **A.** Shows the structure of DPP4 (pdb 1NU8) with bound dipeptide substrate. **B** and **C** show the FAP structure (pdb 1Z68) with di- and tri-peptides docked into the active site. Blue dashed lines highlight potential hydrogen bonding interactions. Conserved substrate-binding residues are labeled in black. The principal residue important for specificity, FAP Ala657/DPP4 Asp663, is labeled in red.

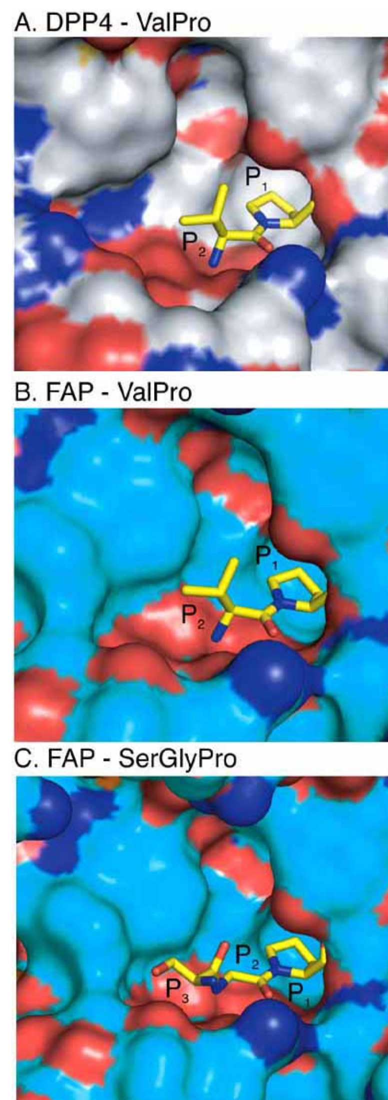

Fig. (5). (**A**) DPP substrate docked into DPP4 (pdb 1NU8). DPP (**B**) and endopeptidase (**C**) substrates modeled into the FAP active site (pdb 1Z68).

with DPP4, POP readily accepted the bulkier N-acyl-Gly-boroPros, consistent with the relative openness of the POP S_3 subsite (Fig. (**6**)). Bachovchin's group recently disclosed a similar series of N-acyl-Gly-boroPros, with **6** being the most potent FAP inhibitor; however, selectivity versus POP was not reported [45]. Sarcosyl- (N-methylglycyl-) analogs of selected acyl-Gly-boroPros (eg. **7**) also inhibited POP and maintained selectivity against DPP4. However, these compounds were less potent FAP inhibitors, possibly due to FAP's narrower S_3 pocket and the proximity of the di-Glu repeat at S_2, which is absent in POP (Fig. (**6**)). N-alkyl-Gly-boroPros (eg. **8**,**9**) reported by Hu *et al.,* showed decreased inhibitory activity against FAP and no selectivity against DPP4, suggesting that selectivity against DPPs requires an N-acyl-substitutent [31]. The N-acyl-blocking group therefore modulates the potency of FAP and POP inhibition and consistently confers selectivity against DPP4.

Additional SAR studies examined a mesyl-N-blocking group (**10**), a P_2 D-ala residue (**11**) and non-peptidic boroPros (eg. **12, 13**) [44]. Both mesyl-Gly-boroPro and acetyl-D-ala-boroPro showed selectivity for FAP relative to DPP4 and POP, but these compounds were less potent than the parental compound (K_i ↑11-15-fold, Table **1**). Non-peptidic boroPros such as **12** and **13** also inhibited FAP and POP, with **12** being the most potent FAP inhibitor of this series. Both compounds lack a terminal amide NH, indicating that FAP and POP inhibition does not require this group. The non-peptidic compounds showed striking selectivity against DPP4, highlighting this protease's marked intolerance for

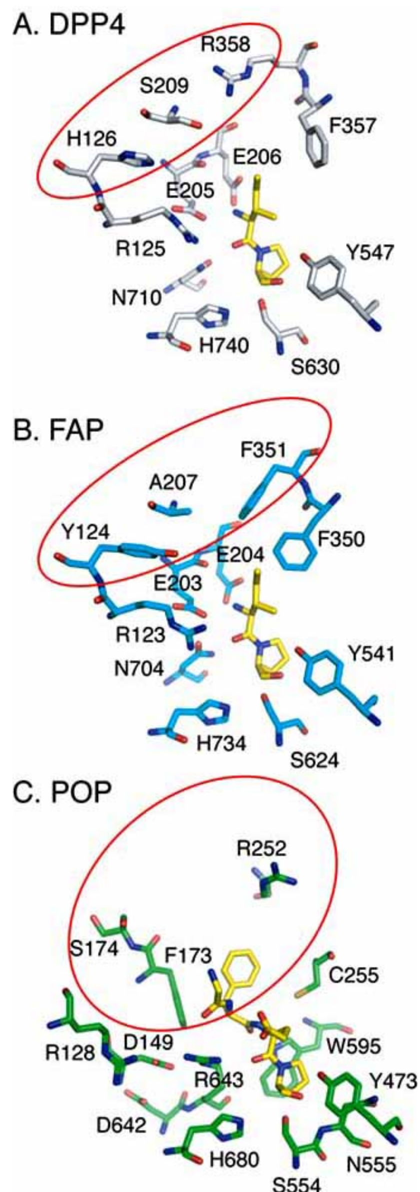

Fig. (6). DPP4 (**A**), FAP (**B**), and POP ((**C**); pdb 1E8M) active sites. The figure models a dipeptide substrate into the FAP active site (pdb 1Z68), based on the DPP4 structure with bound peptide (pdb 1NU8). The red circles highlight non-conserved residues near the S_3 subsite of each protease.

endopeptidase inhibitors and non-peptidic inhibitors lacking a terminal amide NH.

Dipeptide boroPros with a C-substituted α-amino acid at P_2 such as Val-boroPro (**1**), initially developed as DPP4 inhibitors, are also potent FAP inhibitors [29-31, 41]. These compounds are poor POP inhibitors; however, the free amine at P_2 allows these compounds to inhibit other prolyl peptidases with DPP activity including DPPs -7, -8, and -9 [27,29-31]. The free amine additionally allows intramolecular cyclization with the boronic acid moiety, which inactivates the inhibitor. Intramolecular cyclization occurs more readily at higher pHs and depends on the P_2 residue, with Gly-boroPro cyclizing more rapidly than Ala-boroPro, Pro-boroPro and Val-boroPro [29]. In contrast, N-acyl-Gly-boroPros lack the nucleophilic amine at P_2 and therefore do not cyclize. Thus, increased selectivity and chemical stability distinguish the N-acyl-Gly-boroPros from dipeptidic boroPros with a free NH group.

Other Electrophile-Based Inhibitors

Three classes of FAP inhibitors employing a non-boronic acid electrophile coupled to a P_1 pyrrolidine have been described. The first class, 2-cyanopyrrolidines (*aka* cyanopyrroldides, pyrrolidine-2-nitriles, pro-nitriles; Fig. (**7**), Table **2**), employs an electrophilic nitrile at the C2 position of the P_1 pyrolidine as a warhead. 2-cyanopyrrolidines have been extensively studied as DPP4 inhibitors and form an imidate adduct with the active site serine. Interestingly, we found that peptidyl-nitriles such as Val-Pro-CN (**14)**, Ile-Pro-CN and Ac-Gly-Pro-CN (**15)** inhibited FAP much less potently than the corresponding peptidyl-boroPros (300-55,000-fold increase in K_i, Table **2**) [41]. This relates not only to the more electrophilic nature of the boronic acid inhibitors, but also to the relatively less acidic active site of FAP as mutation of FAP Ala657 to Asp as in DPP4 restores high affinity inhibition with Val-Pro-CN and Ile-Pro-CN. Besides simple peptidyl-nitriles, FAP also reacts weakly with cyclohexyl-Gly-Pro-CN (**16**), (5-substituted-pyrrolidinyl-2-carbonyl)-2-cyanopyrrolidines (eg. **17, 18**), 3-[2-((2S)-2-cyano-pyrolidin-1-yl)-2-oxo-ethylamino]-3-methyl-butyramides (**19**), 2-cyano-4-fluoro-1-thiovalylpyrrolidines (eg. **20, 21**) and 5-alkynyl-2-cyanopyrrolidines (eg. ABT-279, **22**), compounds developed as DPP4 inhibitors [46-49]. This is somewhat surprising as these inhibitors contain a lipophilic P_1 constituent and P_2 amine, capable of interacting with FAP's S_1 and S_2 subsites. The less acidic nature of the FAP active site likely contributes to FAP's poor affinity for the aforementioned pro-nitriles, but differences in the FAP and DPP4 S_3 subsites and substitutions on the P_1 pyrroldine ring may also contribute.

Irreversible inhibitors comprise the second and third classes of FAP inhibitors employing non-boronic acid electrophiles. These include peptidyl-chloromethyl ketones (CMK), which likely alkylate the active site histidine and peptidyl-diphenyl phosphonates, which are thought to form a stable phosphoester with the active site serine. CMKs based on endopeptidase substrates, Ac-Gly-Pro-CMK (**23**, Fig. **7**) and Ac-Thr-Ser-Gly-Pro-CMK, inhibited FAP slowly (k_2/K_i = 100-108 $M^{-1}min^{-1}$) and showed no activity against DPP4 [35]. By contrast, diphenyl phosphonates based on DPP substrates (eg. **24**), inhibited FAP with moderate rates of inhibition (k_2/K_i = 0.29-1.2 x 10^4 $M^{-1}min^{-1}$) but lacked selectivity against DPP4 [50, 51]. Interestingly, the corresponding N-blocked (Boc- or Cbz-) phosphonates showed no activity against either protease at concentrations up to 100 μM. Although slow acting, these irreversible inhibitors may serve as probes to monitor protease activity when coupled to a fluorescent detection tag.

SUMMARY AND FUTURE DIRECTIONS

Development of FAP inhibitors has advanced significantly in recent years, but a highly selective inhibitor suit-

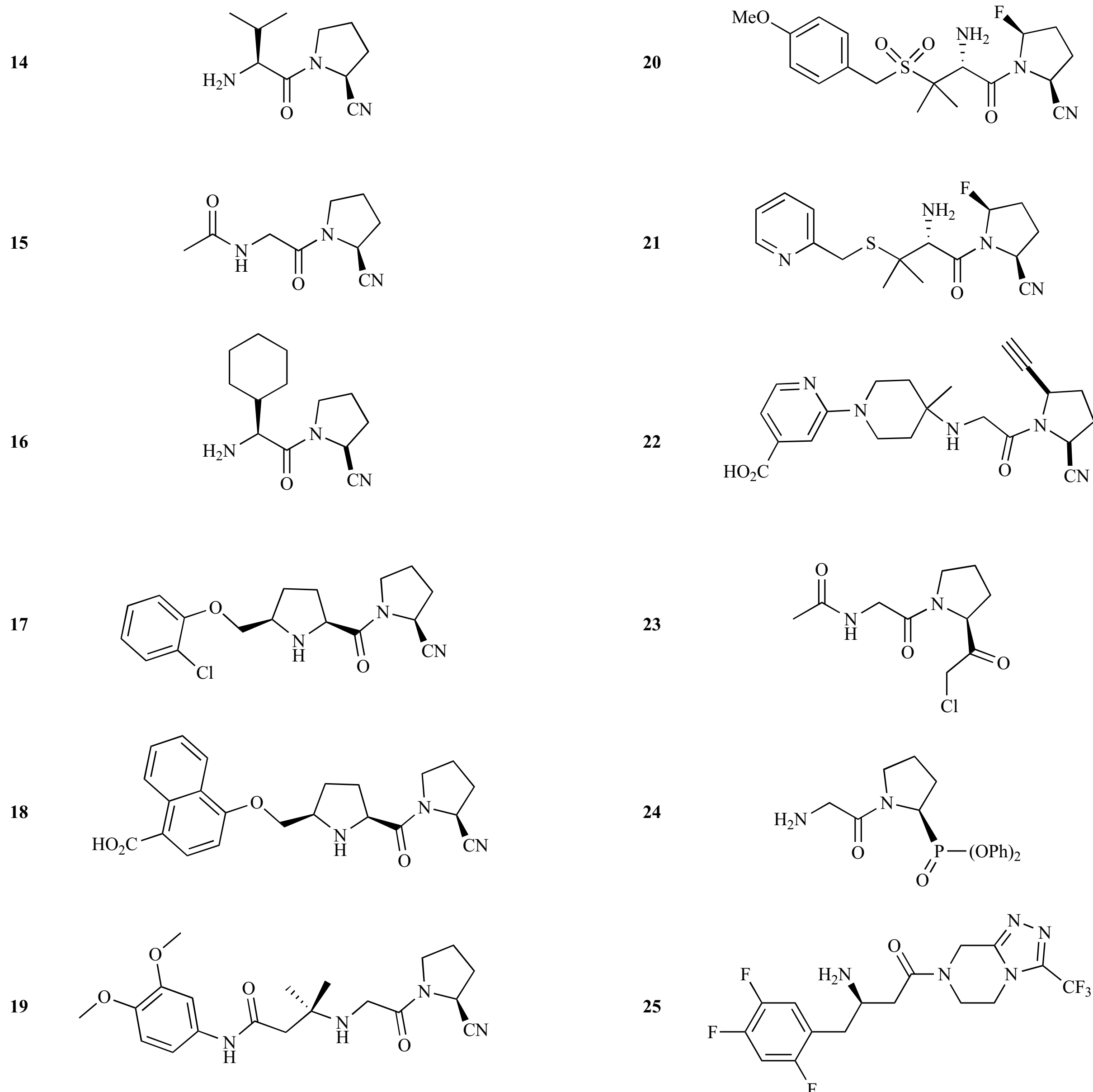

Fig. (7). Additional FAP and DPP4 inhibitors.

able for validating FAP as a therapeutic target has not yet been reported. Inhibitors based on FAP peptide substrate specificity show significant selectivity against DPP4, but lack selectivity against POP. In this regard, Ac-Gly-boroPro (**2**), Ac-D-ala-boroPro (**11**), and mesyl-Gly-boroPro (**10**) provide the most selectivity against POP, but these inhibitors require further optimization. The highly reactive boronic acid present in these inhibitors may limit their *in vivo* selectivity, so further optimization with less electrophilic warheads or P_1 groups lacking an electrophile is desirable. This may be challenging given that FAP reacts poorly with peptidyl-nitriles and DPP4 inhibitors lacking an electrophile such as sitagliptin and derivatives (**25**, Fig. (**7**)), even though they all contain a lipophilic proline-mimetic and free amine capable of interacting with the FAP S_1 and S_2 subsites [41, 52-57]. The less acidic nature of the FAP active site explains much of FAP's poor reactivity with the peptidyl-nitriles and may also contribute to poor binding of inhibitors lacking an electrophile, but this requires further study as differences in the FAP and DPP4 S_3 subsites may also influence inhibitor selectivity. Development of FAP inhibitors lacking an electrophile is therefore a priority for future studies.

A second goal for future studies is to improve inhibitor selectivity against POP and this may be accomplished by at least three complementary methods. First, high throughput screening of small molecule libraries for FAP inhibitors followed by counter-screening of lead compounds against POP may identify novel scaffolds for FAP selective inhibitors. Second, rational inhibitor design may arise from careful analysis of the FAP, DPP4, and POP structures. For exam-

Table 2. Inhibitory Activity (K_i, nM or (IC_{50}, nM)) of Selected Pro-nitriles

Compound	FAP	DPP4	DPP8	DPP9	DPP7	POP	Reference
14	340,000	10.1					[41]
15	6800	61,000					[41]
16	>20,000[1]	1.4					[42]
17	479	1.8	616	107	1545	2762	[46]
18	240	0.96	191	73	5460	925	[46]
19	(>20,000)	(116)	(>20,000)		(>20,000)		[47]
20	> 23,440	53			>22,385		[48]
21	19,076	12			>22,385		[48]
22	>30,000	1.3	>30,000	>30,000	>30,000	>30,000	[49]

[1]Wolf, BB, unpublished data

ple, the FAP S_3 subsite is relatively more hydrophobic than the DPP4 and POP S_3 subsites and contains a Tyr residue (Tyr124) absent in the other two proteases that is capable of hydrogen bonding with inhibitors (Fig. (**6**)). Modification of known endopeptidase inhibitors such as Ac-Gly-boroPro to exploit these S_3 subsite differences might therefore enhance selectivity for FAP. Further clues to selectivity should also arise from solution of a FAP-endopeptidase inhibitor co-crystal structure. Finally, given prior successes with substrate-based design of prolyl peptidase inhibitors, the Substrate Activity Screening (SAS) method might identify new leads for non-peptidic FAP inhibitors [58]. This method initially involves screening the target protease against a non-peptidic fluorogenic substrate library to identify substrate "hits". Based on these hits, focused substrate analogue libraries are then synthesized to identify optimal substrates. The optimal substrates are converted to inhibitors by replacing the fluorescent leaving group of the substrate with an inhibitory warhead. Given the highly successful targeting of DPP4, these approaches should yield FAP selective inhibitors that will allow us to better assess FAP's role in tumorigenesis and validity as a therapeutic target.

ABBREVIATIONS

Ac	=	Acetyl
α_2AP	=	α_2-antiplasmin
boroPro	=	Prolineboronic acid
CMK	=	Chloromethylketone
DPP	=	Dipeptidyl peptidase
FAP	=	Fibroblast activation protein
POP	=	Prolyl oligopeptidase
sarcosyl-	=	N-methylglycyl
SAR	=	Structure-activity relationship
SAS	=	Substrate activity screening

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