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Acylated Gly-(2-cyano)pyrrolidines as inhibitors of fibroblast activation protein (FAP) and the issue of FAP/prolyl oligopeptidase (PREP)-selectivity

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ABSTRACT

A series of N-acylated glycyl-(2-cyano)pyrrolidines were synthesized with the aim of generating structure–activity relationship (SAR) data for this class of compounds as inhibitors of fibroblast activation protein (FAP). Specifically, the influence of (1) the choice of the *N*-acyl group and (2) structural modification of the 2-cyanopyrrolidine residue were investigated. The inhibitors displayed inhibitory potency in the micromolar to nanomolar range and showed good to excellent selectivity with respect to the proline selective dipeptidyl peptidases (DPPs) DPP IV, DPP9 and DPP II. Additionally, selectivity for FAP with respect to prolyl oligopeptidase (PREP) is reported. Not unexpectedly, the latter data suggest significant overlap in the pharmacophoric features that define FAP or PREP-inhibitory activity and underscore the importance of systematically evaluating the FAP/PREP-selectivity index for inhibitors of either of these two enzymes. Finally, this study forwards several compounds that can serve as leads or prototypic structures for future FAP-selective-inhibitor discovery.

Fibroblast activation protein (FAP, FAP-α, seprase) is a Clan SC protease of the prolyl oligopeptidase subfamily, occurring as a cell surface homodimer. FAP has been demonstrated to possess both dipeptidyl peptidase and endopeptidase activity, catalyzed by the same active center. Its expression is associated with activated stromal fibroblasts and pericytes in over 90% of human epithelial tumors examined and with normal or excessive wound healing, for example, tissue remodeling sites or during chronic inflammation. The enzyme is generally not expressed in normal adult tissues and in nonmalignant tumors.[1]

During the last decade, numerous reports have been published that claim an important role for FAP in tumor growth and proliferation and several other pathologic processes that involve degradation of the extracellular matrix.[2] The exact mechanism by which FAP takes part in these processes is unknown, but direct modulation of tumor growth or disease progression by proteolytic processing of growth factors, cytokines, collagenase activity regulating proteins and even collagen derived proteins, is currently the subject of intense research. Several studies have tried to map the physiological substrate spectrum of FAP, including very recent reports that identify for example, α2-antiplasmin, type I collagen and gelatin as in vitro substrates of the endopeptidase activity of FAP.[3] Analogously, Neuropeptide Y, B-type natriuretic peptide, substance P and peptide YY have been identified as in vitro substrates of the dipeptidyl peptidase activity of FAP.[4] Nonetheless, the relevance of these findings under in vivo conditions remains debatable and the unambiguous definition of FAP's physiological substrate spectrum remains a largely untouched matter so far.

While awaiting the detailed functional characterization of the enzyme, several groups currently focus on FAP's status as a potential cancer biomarker whose presence or activity in tumors could also be used for site-directed delivery of oncology drugs.[5] Equally important, FAP or its activity are being targeted by several groups as a direct way to reduce tumor growth and proliferation by means of immunotherapeutic and small molecule inhibitor approaches.[6,7] For the latter, a number of in vivo proof-of-concept studies have been published. These all involve the dipeptide derived boronic acid talabostat (PT-100, Val-*boro*Pro) or close analogues, and report significant activity on tumor stromagenesis and growth.[8] In addition, talabostat has been evaluated as a therapeutic drug in various clinical trials through phase II, for the treatment of, for example, metastatic kidney cancer, chronic lymphocytary leukemia, pancreatic adenocarcinoma and non-small cell lung cancer (Fig. 1). While

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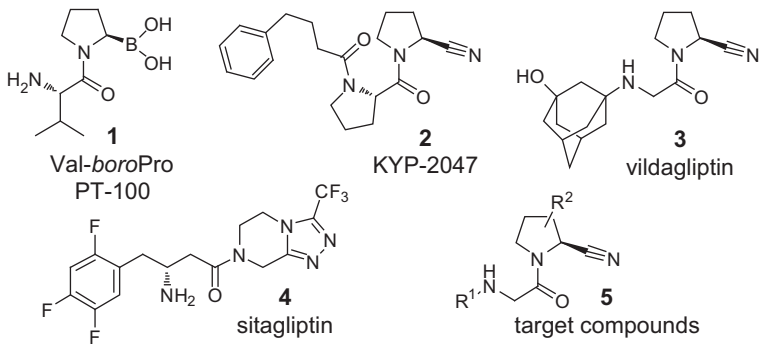


Figure 1. Reference compounds used in this study (**1**–**4**) and generic structure of products reported in this publication (**5**).

talabostat in several of these trials was able to induce clinical responses, questions were raised with regards to the safety profile of the compound, potentially related to its well-known lack of selectivity with respect to other Subfamily S9B proteases.[9]

With the number of reported FAP-inhibitors being small and most of them belonging to the class of boronic acids, we focused on compounds that contain a carbonitrile warhead in place of the boronic acid, but retain an overall dipeptide derived architecture (Fig. 1, generic structure **5**). The latter is a hallmark of most chemotypes of published Subfamily S9B inhibitors. The carbonitrile function itself is also a popular affinity-enhancing moiety in reported series of inhibitors of DPP IV, DPP8, DPP9 and PREP.[10] Compared to other warheads that are used in serine protease inhibitor design (e.g., $-B(OH)_2$, –CHO, chloromethylketones, ketoamides,...) the relatively mildly electrophilic carbonitrile could account for making the inhibitor more selective in vivo, a hypothesis that has been raised in literature earlier.[11]

The common *N*-acyl glycyl-(2-cyano)pyrrolidine scaffold of our compounds was inspired by earlier work from Edosada et al. in which library screening of acetyl-P2-Pro-AMC fluorogenic peptides was used to identify FAP as a protease with particular endopeptidase activity toward acetyl-Gly-Pro sequences.[12] In addition, we anticipated the absence of a basic amino terminus to render compounds with far less affinity for S9B dipeptidyl peptidases, compared to for example, Val*boro*Pro and related inhibitors.[13] As part of this study, two types of modifications were investigated: (1) variation of the *N*-acyl substituent (R^1) and (2) modifications of the (2-cyanopyrrolidine) moiety (R^2) (Fig. 1, generic structure **5**). At the outset of our activities, only isolated cases of carbonitrile inhibitory activities against FAP were reported, mostly in the framework of selectivity assessment of DPP IV inhibitors. Recently, Tsai et al. published a paper that also reports directed investigations aiming at the identification of dipeptide derived carbonitriles as inhibitors of FAP.[14]

All inhibitors were assayed for potency toward FAP, PREP and the dipeptidyl peptidases DPP IV, DPP II and DPP9.[15] Additionally, DPP9 potencies reported can reasonably be expected to be indicative for inhibitor affinities toward the highly homologous DPP8.[16] Furthermore, as was anticipated by taking into account the absence of a basic P2-amine function in the target molecules, these molecules in general do not display measurable affinity for any of the dipeptidyl peptidases tested (vide infra, Tables 2 and 3). Additionally, PREP assay data were considered relevant for this study taking into account the related proline selective endopeptidase activity of the enzyme and the directly related risk of potentially overlapping inhibitor pharmacophores.[17] This is illustrated i.a. by a publication by Tran et al. in which *N*-blocked Gly-*boro*Pro's are presented as dual leads for FAP and PREP inhibitor development.[18]

For a set of representative literature inhibitors of Clan SC enzymes, activities were determined for use as reference standards in this study (Fig. 1, Table 1). This set consists of Val-*boro*Pro, the aforementioned, non-selective boronate that has been extensively applied for i.a. in vitro and in vivo blocking of FAP activity.[8,9] Inhibitor KYP-2047 (**2**) can be regarded as a selective PREP-inhibitor with respect to the set of target enzymes tested, notwithstanding the fact that a prolylpyrrolidine skeleton is present in several compounds that were described to possess FAP- and DPP-affinity.[11,19] The clinically used DPP IV inhibitors sitagliptin (**3**) and vildagliptin (**4**) were also found to lack FAP affinity.

The first set of inhibitors synthesized in the framework of this study, differ by variation in the *N*-acyl residue (R_1 in generic structure **5**, R_2 = H) (Table 2, **26** and **27**). All compounds in this series were prepared by acylation of the amino terminus of Gly-(2-cyano)pyrrolidine, either by reaction with commercially available acyl chlorides (or sulfonyl chloride in **20**), or by TBTU-mediated coupling using the appropriate carboxylic acid. A considerable number of compounds in Table 2 on the other hand, display dual FAP and PREP affinity with often roughly comparable IC_{50}-values. Only compounds **21**, **22**, **26** and **27** possess inhibitory profiles in which substantial FAP potency (IC_{50} <5 μM) is decoupled from PREP binding potential. The common structural feature that potentially accounts for this profile, is an (azaheterocyclyl)acetyl group as the *N*-acyl scaffold substituent. Certainly, both the scope of this claim and the possibility to improve FAP affinity by further exploration of this structural characteristic, should be the subject of fur-

Table 1
Affinity data for reference compounds **1**–**4**

Compd	IC_{50} (μM)				
	FAP	PREP	DPP II	DPP IV	DPP9
1	0.066 ± 0.011	0.98 ± 0.06	0.086 ± 0.007	0.022 ± 0.001	n.d.[a]
2	>100	0.006 ± 0.004	>100	>100	>100
3	52 ± 18	>100	>1000	0.12 ± 0.001	0.68 ± 0.02
4	>100	>100	>100	0.04 ± 0.001	>100

Table 2
Acylglycyl-(2-cyanopyrrolidine) based inhibitors: variation of the acyl moiety (R^1 in generic structure **5**, R^2 = H)

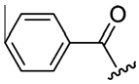
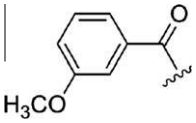
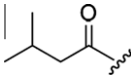
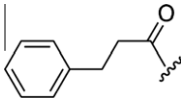
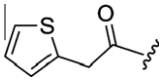
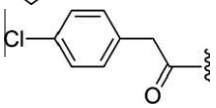
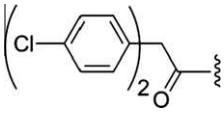
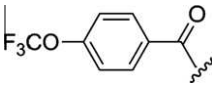
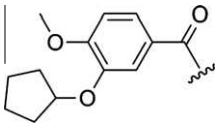
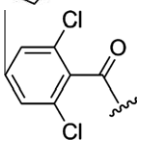
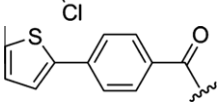
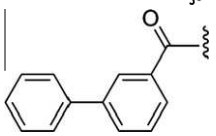
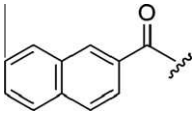
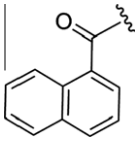
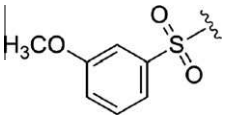
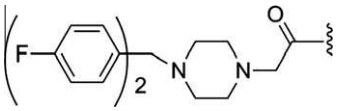
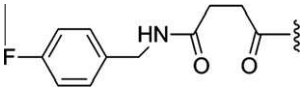
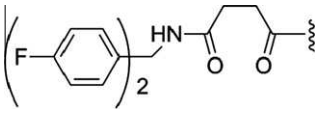
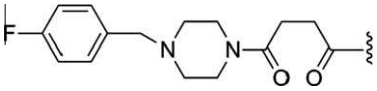
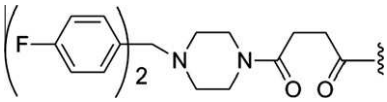
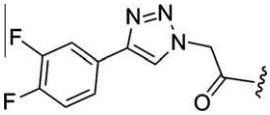
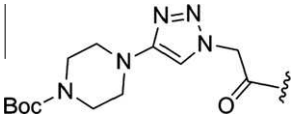
Compd	R^1	IC_{50} (μM)				
		FAP	PREP	DPPII	DPPIV	DPP9
6		3.5 ± 0.1[a]	10.7 ± 0.5	>100[a]	>100	>100
7		2.4 ± 0.1	15.9 ± 0.9	>100	>100	>100
8		14 ± 0.4	9.4 ± 1.1	>100	>100	>100
9		9.4 ± 0.4	1.6 ± 0.1	>100	>100	>100
10		6.8 ± 0.2	2.6 ± 0.2	>100	>100	>100
11		3.9 ± 0.2	0.60 ± 0.03	>100	>100	>100
12		1.9 ± 0.1	2.9 ± 0.1	>100	>100	>100
13		4.7 ± 0.2	5.0 ± 0.2	>100	>100	>100
14		3.7 ± 0.2	>10	>100	>100	>100
15		14.6 ± 0.5	>100	>100	>100	>100
16		8.1 ± 0.2	1.7 ± 0.2	>100	13.1 ± 0.7	20.2 ± 1.3
17		1.4 ± 0.1	5.8 ± 0.6	>100	>100	>100
18		7.5 ± 0.6	5.4 ± 0.4	>100	>100	>10
19		0.67 ± 0.04	3.3 ± 0.2	>100	>100	>10
20		5.1 ± 0.5	>10	>100	>100	>100

Table 2 (*continued*)

Compd	R^1	IC_{50} (μM)				
		FAP	PREP	DPPII	DPPIV	DPP9
21		2.7 ± 0.1	>10	>100	>100	>100
22		12 ± 1	>50	>100	>100	>100
23		20 ± 1	>50	>100	>100	>>100
24		10.3 ± .5	>50	>100	>100	>100
25		19.9 ± 1.3	>50	>100	>100	>100
26		1.3 ± 0.1	>50	>100	>100	69 ± 2
27		2.7 ± 0.1	>100	>100	>100	>100

[a] '>' means that residual enzymatic activity is higher than 50% at the indicated concentration.

ther investigation. Finally, the presence of a sulfonyl instead of an acyl linkage (**7** vs **20**) does not seem to have significant implications for either FAP affinity of FAP/PREP selectivity.

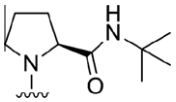
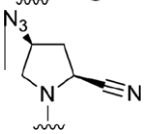
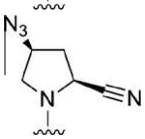
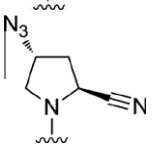
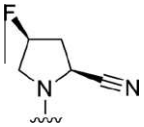
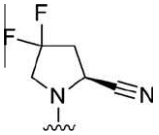
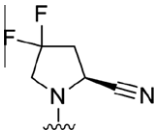
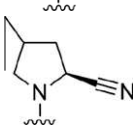
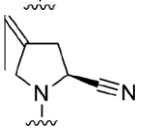
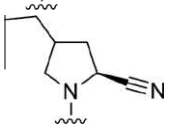
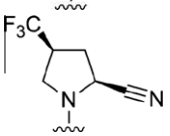
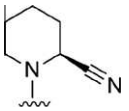
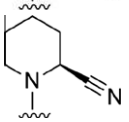
Regardless of the selectivity issue, extracting structure–activity relationship (SAR)-data from Table 2 with specific regards to affinity for FAP was another primary goal of this study. The P3-region of FAP, in which the *N*-acyl susbtituents can be expected to be accommodated, clearly does not impose strict requirements with respect to steric bulk: even large substituents still allow binding of the inhibitors, as demonstrated by compounds **12** and **14**–**27**. More detailed information on the available space in the P3 region can be derived by comparing the rigid and bulky regio-isomeric inhibitor pairs **16** and **17** (the former containing a thiophene ring as an isosteric benzene replacement) and **18** and **19**. Both indicate FAP's preference of almost an order of magnitude for the compounds in which the distal part of the ring system is in a skewed position relative to the acyl group. In addition, compound **19**, containing a 1-naphthoyl substituent, was found to be the most potent inhibitor in this series. Our selection of the 1-naphthoyl residue was based on a patent by Bachovchin and Lai in which the activity of *N*-(1-naphthoyl)-substituted Gly-*boro*Pro was claimed to possess superior FAP-affinity relative to the *N*-benzoyl substituted congener, an observation we found to also hold for the corresponding nitriles.[20] In addition, compound **19** was also reported in the aforementioned publication by Tsai et al. with comparable FAP potency, but not including PREP assay data.[14]

In a second compound series, the influence of modifications at the pyrrolidine ring was investigated (R^2 in generic structure **5**, R^1 = benzoyl or 1-naphthoyl, results summarized in Table 3). All

sponding pyrrolidine carboxamide (or pyrrolidine in the case of **28**). Dehydration of the carboxamide group using trifluoroacetic acid/pyridine was used to install the carbonitrile group. The preparation of the different pyrrolidine carboxamides used in this study was achieved based on literature procedures.[21] From the evaluation results of inhibitors **28** and **29** (compared to **6**), it is clear that the carbonitrile group significantly contributes to FAP affinity. This observation is indicative for the interaction of the enzyme's catalytically active serine-OH with the carbonitrile–carbon, potentially involving the formation of an enzyme-bound imidate, as has been demonstrated for example, DPP IV by X-ray crystallography.[22]

In addition, subsitution of the (2-cyano)pyrrolidine group at the 4-position, was explored. Earlier reports from our group have indicated that introduction of substituents at this position can lead to significant increase of enzyme affinity in P1-pyrrolidine containing inhibitors of DPP IV and DPP8/9.[23] In the case of FAP however, available space at this position seems very limited. First, compounds **30–32** that contain either a 4*R* or 4*S*-azido substituent clearly display lower inhibitory activity compared to their non-substituted congeners **6** and **19**. The 4*R*-azido substituent in **32** even seems not to be accepted by the enzyme. Likewise, results measured for 4-methyl-, methylene-, ethyl- and 4*R*-trifluoromethyl- substituted analogues consistently are indicative of the same conclusion. Notably, this effect is least pronounced for the 4-methylene substituted analogue **37**, which can be expected to also impose substantial conformational constraints on the pyrrolidine ring system. Further, fluorinated inhibitors **33–35**, are the only compounds from Table 3 that outperform FAP-potency of their non-substitued analogues with no significant difference ob-

Table 3
P1-modifications studied

Compd	P1	R^1	IC_{50} (μM)				
			FAP	PREP	DPPII	DPPIV	DPP9
28		Benzoyl–	>100[a]	—	>>100	>100	>10[b]
29		Benzoyl–	>25	>100	>100	>100	>10
30		Benzoyl–	17.5 ± 0.7	>100	>100	>100	>10
31		1-Naphthoyl–	4.1 ± 0.4	>100	>100	>100	>10
32		1-Naphthoyl–	>100	>100	>100	>100	>10
33		1-Naphthoyl–	0.126 ± 0.007	1.1 ± 0.2	>100	>100	>10
34		Benzoyl–	0.85 ± 0.07	>10	>100	>100	>10
35		1-Naphthoyl–	0.110 ± 0.007	4.84 ± 0.4	>100	>100	>10
36		Benzoyl–	>100	>100	>100	>100	>10
37		1-Naphthoyl–	6.7 ± 0.4	>100	>100	>100	>10
38		1-Naphthoyl–	42 ± 3	>100	>100	>100	>10
39		1-Naphthoyl–	>100	>100	>100	>100	>10
40		Benzoyl–	>100	>100	23.3 ± 0.4	>100	>10
41		1-Naphthoyl–	>50	>100	>100	>100	>10

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