Advanced Fibroblast Activation Protein-Ligand Developments

FAP Imaging Agents: A Review of the Structural Requirements

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KEYWORDS

• Seprase • FAP • Boroproline • Cyanoproline • Cancer-associated fibroblasts

KEY POINTS

- Small molecule inhibitors of fibroblast activation protein-α (FAP) are being developed for radioimaging of FAP expression in humans.
- Small molecule inhibitors of FAP are being developed for targeted radiotherapy of tumors in humans.
- Cyanoproline and boroproline containing FAP inhibitors can be exploited as imaging agents and targeted radiotherapeutics.

INTRODUCTION

Fibroblasts are one of the most abundant cell types in connective tissues. These cells are responsible for tissue homeostasis under normal physiological conditions. When tissues are injured, fibroblasts become activated and differentiate into myofibroblasts, which generate large contractions and actively produce extracellular matrix (ECM) proteins to facilitate wound closure. Both fibroblasts and myofibroblasts play a critical role in wound healing by generating traction and contractile forces, respectively, to enhance wound contraction.

The tumor microenvironment comprises tumor cells and a heterogeneous mix of accessory cells (the tumor stroma), which are critical to tumor development.[1,2] The tumor stroma includes fibroblasts, epithelial cells, endothelial and smooth muscle cells of the vasculature, fat, and immune cells.[3] Although these cells are not malignant, they acquire an altered phenotype allowing them to support and enhance tumor growth. Activated cancer-associated fibroblasts (CAFs) are the primary cellular component of the tumor stroma and have been shown to assist in cancer progression by upregulating the expression of several proteins, including growth and chemotactic factors, angiogenic factors, and matrix metalloproteases.[1–3]

Fibroblast activation protein-α (FAP), also known as seprase (*s*urface *e*xpressed *pr*otease), is a membrane dipeptidyl peptidase (DPP) of the family of serine proteases. FAP expression is normally restricted to fetal mesenchymal tissue; however, it is selectively expressed in reactive stromal fibroblasts of epithelial cancers and in dermal scars of healing wounds,[4] as well as in liver cirrhosis.[5] The majority of FAP is expressed by activated fibroblasts responding to pathologic situations. FAP is a 170 kDa, type II, integral membrane peptidase in the dipeptidyl peptidase-4 (DPPIV) family of prolyl peptidases, originally defined as the target of a mouse monoclonal antibody, F19.[6] As a type II transmembrane protein, FAP is typically found physically attached to cells

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and with the bulk of the protein, including the catalytic domain, exposed to the extracellular space and accessible to small molecules. Small amounts of soluble FAP are also found in circulation in humans and other mammals.[7,8]

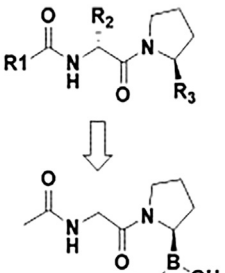
FAP is a nonclassical serine protease, which belongs to the S9B prolyl oligopeptidase subfamily. FAP is most closely related to DPPIV (approximately 50% of their amino acids are identical). The active site of FAP is localized in the extracellular part of the protein and contains a catalytic triad composed of Ser^{624}, Asp^{702}, and His^{734} in humans and mice.[9] FAP is catalytically active as a 170-kD homodimer and has a dipeptidase and an endopeptidase activity. During the last 2 decades, FAP has attracted increasing attention as a selective marker of CAFs and, more broadly, of activated fibroblasts in tissues undergoing remodeling of their ECM due to chronic inflammation, fibrosis, or wound healing.

FAP is a key component of the tumor microenvironment.[10] A highly consistent feature of tumor stromal fibroblasts or CAFs is the induction of FAP. FAP is a candidate as a universal target antigen because it is reported to be selectively expressed in nearly all solid tumors by a subset of tumor stromal fibroblasts.[11–13] In addition, FAP is expressed on invadopodia of some human breast cancers[14] and melanomas,[15,16] including the human malignant melanoma, LOX, from which it was first identified.[15] Cancer cells that overexpress FAP exhibit an invasive phenotype,[14] serum-free growth,[17] enhanced growth and metastasis in vivo,[18] and exhibit greater microvessel density in the tumor microenvironment.[14] Abrogation of CAF FAP enzyme activity, either through mutagenesis[19] or with a neutralizing antibody,[20] attenuates tumor growth. In summary, CAFs are a dynamic component of the tumor microenvironment that provides mechanical support and controls proliferation and survival, angiogenesis, metastasis, immunogenicity, and resistance to therapies.[21–23]

FAP inhibitors as radioligands for imaging and therapy: The selective expression of FAP on CAFs makes it an attractive target to exploit for noninvasive tumor imaging as well as targeted radiotherapy of via tumor stroma. Clinical trials conducted with [I-131]F19[6] and an [I-131]-radiolabeled humanized version of F19, sibrotuzumab,[24] have demonstrated selective tumor uptake and minimal normal tissue retention in patients with colorectal cancer,[24] thus validating FAP as a molecular target for radioscintigraphy. Although intact antibodies such as sibrotuzumab offer potential for tumor radiotargeting, long circulating

effectiveness as radiodiagnostic and radiotherapeutic agents.

FAP is an atypical serine protease that has both dipeptidyl peptidase and endopeptidase activities, cleaving substrates at a postproline bond. FAP possess both prolyl dipeptidyl peptidase[25,26] as well as gelatinase activity,[16] and a variety of inhibitors of the former catalytic activity has been described.[25–28] Most of these compounds are based on PT-100, a Val-boro-Pro analog, which exhibits antitumor activity in tumor-bearing mice.[28] Although inhibitors of this class are potent, they also block the exopeptidase activity of several other DPPIV family members.[25] To overcome this lack of selectivity, Wolf and colleagues designed compounds, which incorporated a chemical cap on the amino terminus of a Gly-boro-Pro dipeptide.[26] One of their lead compounds, Ac-Gly-boro-Pro, (**Fig. 1**), continues to be recognized by FAP, which displays both exopeptidase and endopeptidase activities but not by several other DPPIV family members that have no endopeptidase activity.[25] In addition, this group demonstrated that potent anti-FAP activity, and selectivity is maintained when significant bulk is added to the amino terminus of Gly-boro-Pro.[26] These observations suggest that it is possible to substitute the amino terminus of Gly-boro-Pro with a chelator capable of coordinating a radioactive metal while retaining affinity for



Ac-Gly-boro-Pro

Protease	Ki (nM)	Selectivity
FAP	23	1
DPP-4	377	16.4
DPP-8	19,100	830
DPP-9	8,800	383
APH	575	25
POP	211	9.2
DPP-7	125,000	5434

Fig. 1. Design of R-AA-Pro inhibitors of FAP. TOP: core structure. Middle: Ac-Gly BoroPro. Bottom: Affinity

FAP, resulting in a radiopharmaceutical that targets FAP in cancer-associated stromal cells for the potential diagnosis and treatment of cancer.

The activities and specificities of FAP have been investigated using artificial substrates and synthetic peptide libraries,[25,29–32] which revealed a strong preference for FAP cleavage of endopeptidase substrates after glycine-proline (Gly-Pro) motifs. A major hurdle in the study of FAP enzyme activity has been the lack of selective inhibitors against this protease. FAP shares DPP specificity with the enzyme members of the DPP4 family, DPP4, DPP8, and DPP9, as well as endopeptidase specificity with prolyl endopeptidase (PREP). Thus, designing small molecule inhibitors that are selective for FAP over other DPPs and prolyl endopeptidase (PREP) presents a challenge.

Radiolabeled FAP Inhibitors. In 2009, a series of radioiodinated FAP inhibitors for targeting the tumor microenvironment was described based on a series of iodine substituted benzamido-glycine-boronoproline analogs (**Fig. 2**).[33] Iodine was substituted at the 3 positions of the benzene ring, and compounds were assessed for their ability to inhibit the enzymatic activity of recombinant human FAP in a fluorescence-based assay. Among the most active compounds, an ortho-iodine analog (MIP-1231) displayed an IC50 of 6 nM, whereas even more potent para-substituted (MIP-1232) and meta-substituted (MIP-1233) analogs both had IC50 values of 0.6 nM. To examine the selectivity for FAP over other prolyl peptidases, compounds were tested for their ability to inhibit the enzymatic activity of PREP. The IC50 values of MIP-1231, MIP-1232, and MIP-1233 for PREP were 58, 19, and 7 nM, respectively, with PREP/FAP ratios of 10, 32, and 12, respectively. These data demonstrate that although the para-substituted and meta-substituted compounds have a similar ability to inhibit FAP activity, the para-substituted analog displayed better selectivity. To examine binding to FAP in vivo, human embryonic kidney (HEK-293) cells were stably transfected with the human FAP gene. The equilibrium dissociation constant (Kd) of MIP-1232 for FAP was determined to be 30 nM, whereas there was no specific binding to a nonexpressing clone. The Bmax of the FAP-expressing cells was determined to be approximately 8 pmol/10^6 cells. In addition, MIP-1232 was shown to inhibit the FAP enzymatic activity of the stable FAP-expressing cells. The authors conclude then that radiolabeled FAP inhibitors could be exploited for the diagnosis, staging, prognosis, and potential treatment of solid tumors. Subsequently, Meletta and colleagues described the use of radioiodinated MIP-1232, for its potential to image atherosclerotic plaques.[34] Ex vivo autoradiography showed strong specific accumulation of radiolabeled FAP inhibitor in FAP-positive SK-Mel-187 melanoma xenograft tissue slices while accumulation was negligible in NCI-H69 xenograft tissue slices with low-FAP levels. Binding of the tracer was similar in plaques and normal arteries, hampering its use for atherosclerosis imaging.

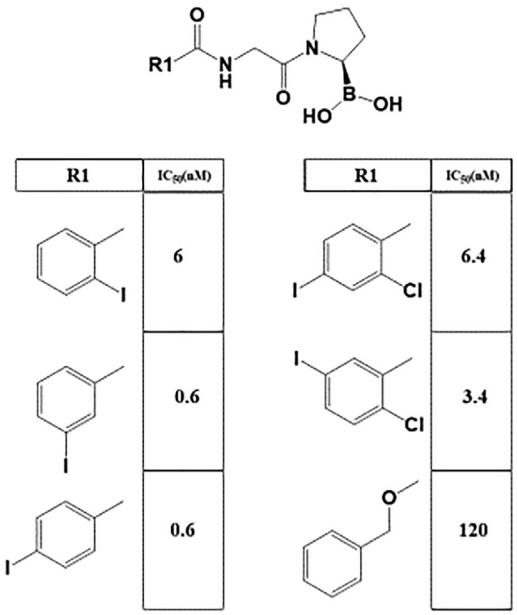


Fig. 2. Iodinated Gly-BoroPro inhibitors display high affinity for FAP. (*Adapted from* Marquis J, Wang J, Maresca K, Hillier G, Zimmerman C, Joyal J, Babich J; Abstract #4467: Targeting tumor microenvironment

In 2013, 2 independent groups reported on the development of potent FAP-selective inhibitors. Poplawski and colleagues reported on potent FAP-selective and PREP-selective inhibitors using boroproline-based compounds (**Fig. 3**).[35] One compound, N-(pyridine-4-carbonyl)-D-Ala-boro-Pro, has a more than 350-fold selectively for FAP over PREP, and with negligible potency against DPP4, DPP8, and DPP9. The University of Antwerp reported on a new class of FAP inhibitors based on an N-(4-quinolinoyl)-Gly-(2-cyanopyrolidine) scaffold.[36] Of the 34 compounds reported on in that study (**Fig. 4**), compound 7 has particular selectivity toward FAP over the related proteases DPP4, DPP8, and DPP9, and is also selective over PREP. Modifications to the quinolinoyl ring improved the selectivity for FAP over PREP,[36] indicating that further developments of this scaffold can yield compounds with more selectivity for FAP.

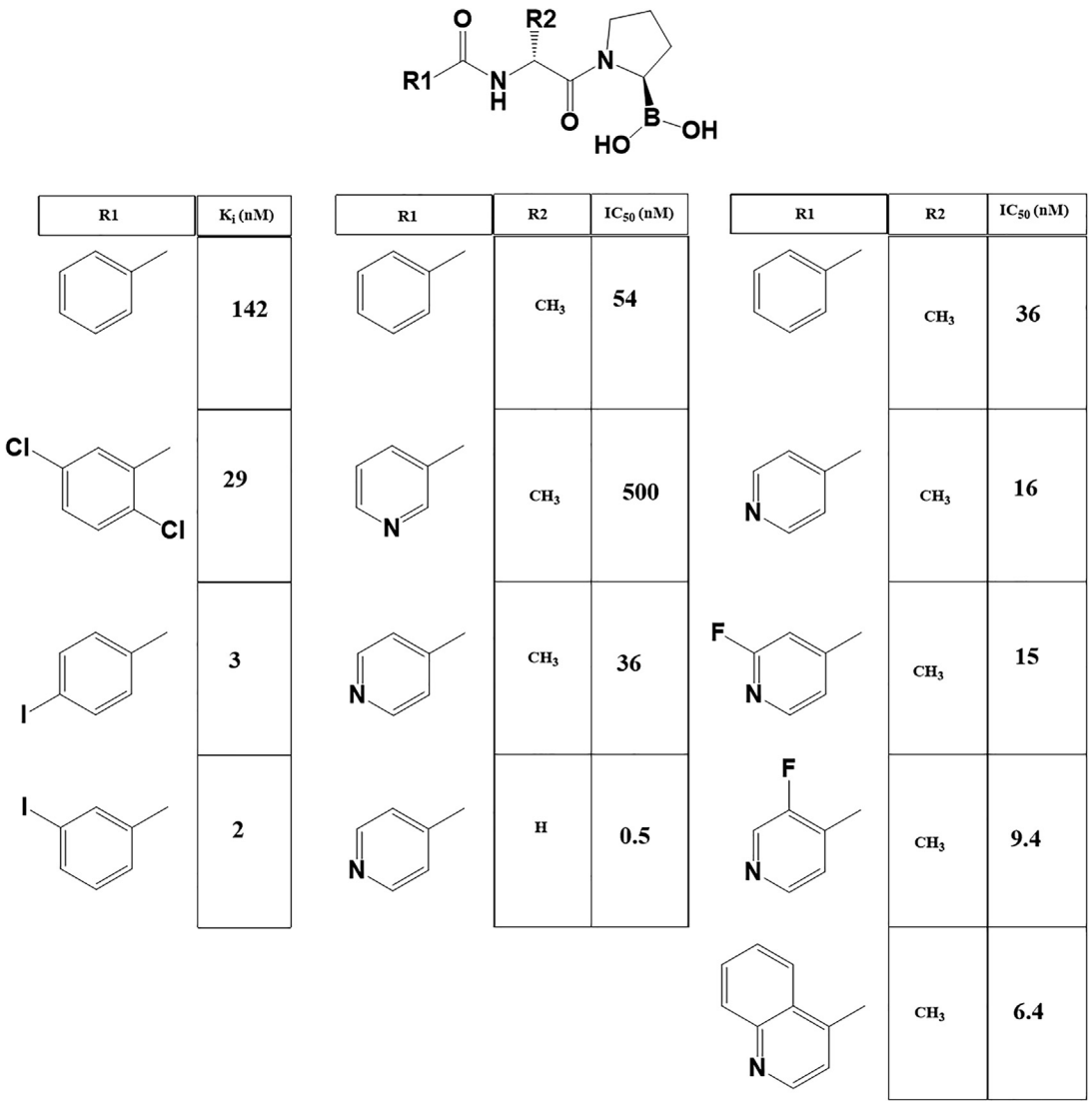


Fig. 3. Structural requirements for FAP affinity and selectivity in Ar-(CO)-D-Ala-BoroPro. (*Data from* Refs.[26,33,35])

Heidelberg group who reported[37] on 2 radioligands based on the N-(4-quinolinoyl)-Gly-(2-cyanopyrolidine) scaffold previously reported by Jansen and colleagues[36] including an iodinated derivative and a derivative containing the chelate DOTA (2, 2′,2″,2‴-[1,4,7,10-Tetraazacyclododecane-1,4,7, 10-tetrayl] tetraacetic acid) that could potentially be labeled with various radiometals. When the DOTA derivative was labeled with gallium-68 ($t_{1/2}$ = 68 min), this radiolabeled FAP inhibitor (FAPI-02) clearly delineated tumors in animals and in humans with 28 unique cancers being visualized in patient studies. [Ga-68]-FAPI-02 achieved excellent contrast to background as the activity localized only to tumor tissue and was quickly excreted via the renal excretion pathway.[38,39] Since this initial exploring FAP radiopharmaceuticals for imaging and therapy applications.[40] The remainder of this article will attempt to provide some insights into the structure activity relationship (SAR) of these small molecule inhibitors.

RATIONALE FOR FIBROBLAST ACTIVATION PROTEIN-α INHIBITOR DESIGN

FAP is a serine protease with Gly2-Pro1-cleaving specificity.[41] Wolf and coworkers demonstrated that dipeptides in which Gly was replaced by D-Ala or D-Ser remained substrates for FAP but that the enzyme was highly specific for proline.[30] Early structure-based designs of FAP inhibitors retained these key structural elements and added a third

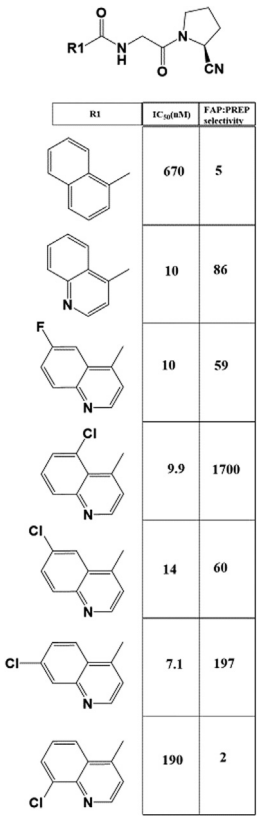


Fig. 4. Impact of aromatic substitution on FAP:PREP selectivity. (*Data from* Jansen K, Heirbaut L, Cheng JD, Joossens J, Ryabtsova O, Cos P, Maes L, Lambeir AM, De Meester I, Augustyns K, Van der Veken P. Selective Inhibitors of Fibroblast Activation Protein (FAP) with a (4- Quinolinoyl)-glycyl-2-cyanopyrrolidine Scaffold. ACS Med Chem Lett. 2013 Mar 18;4(5):491-6.)

specificity. The first potent (23 nM) FAP inhibitor of this class (see **Fig. 1**), Ac-Gly-BoroPro, had good-to-modest selectivity versus other proline-selective peptidases (DPP-4, DPP-7, DPP-8, DPP-9, acyl-peptide hydrolase (APH), and PREP).[25]

Although FAP inhibitors featuring alternative pharmacophores have been prepared, compounds exhibiting the substitution pattern highlighted in **Fig. 1** (R_1 = aromatic, R_2 = H or CH_3, and R_3 = $B(OH)_2$ or CN) are most relevant to potential radiotracer development. The parameter space for each of the key design elements ($R_{1\text{-}3}$) in **Fig. 1** has been probed by medicinal chemistry; potency and selectivity data will be discussed below. These previously published data have been sifted and sorted to emphasize specific trends in the SAR.

Results in **Fig. 3** (left column) from Genentech[26] and Molecular Insight[33] suggest that the R_1 position can accommodate large arenes, and that FAP polarizability. Data in **Fig. 3** (middle column)[35] demonstrate quite convincingly that site-specific (4-position) incorporation of a nitrogen atom in the aryl R_1-substituent results in an increase in potency, which is modest for alanine and quite dramatic in the glycine case. In contrast, a ~14-fold loss in affinity is seen on changing the 4-pyridyl substituent to 3-pyridyl, perhaps indicating that a specific interaction of the 4-pyridyl nitrogen, hypothesized to involve a hydrogen bond to Glu^{204} in the FAP active site,[35] overcomes the loss of arene polarizability caused by endocyclic nitrogen. The FAP-inhibition data provided in **Fig. 3** (right column) suggest that the trends seen in the first 2 columns of **Fig. 3** are additive; increasing the size and/or polarizability of the heterocycle increases FAP inhibition. Data collated in **Fig. 4** show that the R_1-substituent properties that increase inhibition in the boronic acid series (R_3 = $B(OH)_2$, see **Fig. 3**) lead to similar effects in the cyanoproline series (CNPro, R_3 = CN, see **Fig. 4**).[36] Once again, introduction of a fluorine atom into the heterocyclic core (see **Fig. 4**, entry 3, 4-(6-fluoroquinolyl) substituent) is well tolerated and occurs without loss of inhibition (although with some decline in selectivity). The similar inhibition observed for the 5-choroquinoloyl, 6-choroquinoloyl, and 7-choroquinoloyl substituents suggests that fluorine substitution at these locations should lead to potent and selective FAP inhibitors.

Finally, it should be noted that a change in R_2 results in diverging trends in the boronic acids and cyano derivatives. Replacement of D-Ala for glycine in the pyridyl and quinolyl series of boronic acids (see **Fig. 3** middle column) leads to a loss of affinity but an increase in selectivity versus similar proteases.[35] In contrast, Gly → D-Ala substitution is associated with an increase in potency and a decrease in selectivity in the 2-cyanopyrrolidine series (data not shown).[36]

IMPROVING RETENTION

For radiotherapeutic applications, tumor retention is a key parameter that is yet to be optimized. Most small molecule inhibitors of the cyanoproline class show relatively rapid renal clearance and modest tumor retention. Moon and coworkers[42] conducted a head-to-head comparison of a dimeric FAP inhibitor [^{68}Ga]Ga-DOTAGA.$(SA.FAPi)_2$ with monomeric [^{68}Ga]Ga-DOTA.SA.FAPi. Inhibition measurements revealed excellent affinity and selectivity with low nanomolar IC50 values for FAP. In PET/computed tomography human studies, significantly higher tumor uptake as well as longer tumor retention could be observed for the dimer. In first in human studies, Ballal and coworkers reported that [^{177}Lu]

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