

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 (surface expressed protease), 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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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 (K_d) of MIP-1232 for FAP was determined to be 30 nM, whereas there was no specific binding to a nonexpressing clone. The B_{max} of the FAP-expressing cells was determined to be approximately 8 pmol/10⁶ 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.

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 selectivity 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-cyanopyrrolidine) 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.

The pursuit of small molecule inhibitors of FAP as radioligands was bolstered in 2018, by the

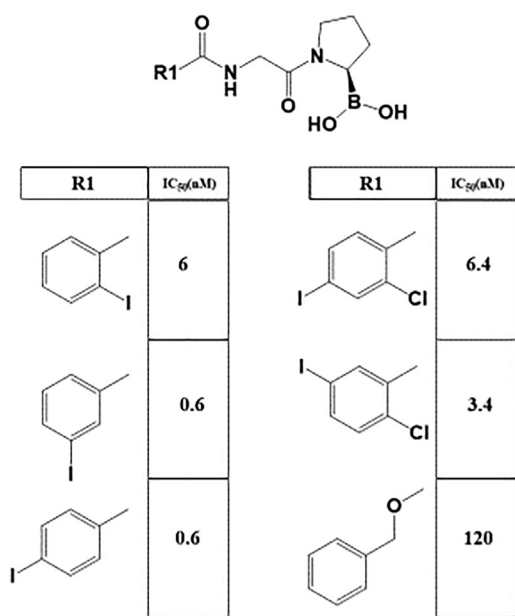


Fig. 2. Iodinated Gly-BoroPro inhibitors display high affinity for FAP. (Adapted from Marquis J, Wang J, Maresca K, Hillier G, Zimmerman C, Joyal J, Babich J; Abstract #4467: Targeting tumor microenvironment with radiolabeled inhibitors of seprase (FAP \#945). Cancer Res 1 May 2009; 69 (9_Supplement): 4467.)

R1	K _i (nM)	R1	R2	IC ₅₀ (nM)	R1	R2	IC ₅₀ (nM)
	142		CH ₃	54		CH ₃	36
	29		CH ₃	500		CH ₃	16
	3		CH ₃	36		CH ₃	15
	2		H	0.5		CH ₃	9.4
						CH ₃	6.4

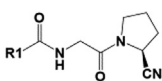
Fig. 3. Structural requirements for FAP affinity and selectivity in Ar-(CO)-D-Ala-BoroPro. (Data from Refs.^{26,33,35})

Heidelberg group who reported³⁷ on 2 radioligands based on the N-(4-quinolinoyl)-Gly-(2-cyanopyrrolidine) scaffold previously reported by Jansen and colleagues³⁶ 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 publication, there has been dramatic growth in

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 group to extend the peptide chain and provide



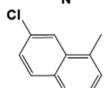
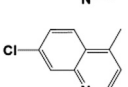
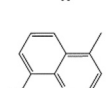
R1	IC ₅₀ (nM)	FAP:PREP selectivity
	670	5
	10	86
	10	59
	9.9	1700
	14	60
	7.1	197
	190	2

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 = $\text{B}(\text{OH})_2$ or CN) are most relevant to potential radiotracer development. The parameter space for each of the key design elements (R_{1-3}) 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 Genentech²⁶ and Molecular Insight³³ suggest that the R_1 position can accommodate large arenes, and that FAP affinity is correlated with arene size and/or

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 Glu²⁰⁴ 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 = $\text{B}(\text{OH})_2$, 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-chloroquinolyl, 6-chloroquinolyl, and 7-chloroquinolyl 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 $\rightarrow$ 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 coworkers⁴² conducted a head-to-head comparison of a dimeric FAP inhibitor [⁶⁸Ga]Ga-DOTAGA.(SA.FAP)₂ with monomeric [⁶⁸Ga]Ga-DOTA.SA.FAP. 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 [¹⁷⁷Lu] Lu-DOTAGA.(SA.FAP)₂ exhibited significant tumor

retention out to 168 hours in a patient with a follicular variant of papillary carcinoma; accumulation in the normal organs peaked during 24 to 48 hours and decreased significantly by 96 hours after injection.⁴³ In similar studies, Zhao and coworkers⁴⁴ synthesized a Ga-68-labeled FAPI dimer ⁶⁸Ga-DOTA-2P(FAPI)₂ and demonstrated improved potency and tumor retention in preclinical models and in patients with cancer compared with FAPI-46. Zhao and coworkers⁴⁵ synthesized the Lu-177 derivative [¹⁷⁷Lu]Lu-DOTA-2P(FAPI)₂ and demonstrated that improved tumor retention was seen out to 48 hours in a hepatocellular cancer patient-derived xenograft (HCC-PDX) and HT-1080-FAP mouse models. Although the dimer approach seems to result in improved retention, it is not yet clear that uptake and tumor retention are sufficient for effective Lu-177 radiotherapy with these constructs.

Cyclic peptide library screens have identified several FAP-binding radiotherapy candidates that exhibit potential improvements in tumor retention. In first in human studies, Baum and coworkers examined biodistribution and preliminary dosimetry in radiotherapy of adenocarcinomas using [¹⁷⁷Lu]Lu-FAP-2286.⁴⁶ In several patients, retention of [¹⁷⁷Lu]Lu-FAP-2286 in metastases was seen out to 10 days after injection. Treatment with 2.4 GBq of [¹⁷⁷Lu]Lu-FAP-2286 resulted in a mixed response after 8 weeks—regression of bone and bone marrow lesions—but overall progressive disease with new evidence of liver metastases. [¹⁷⁷Lu]Lu-FAP-2286 is now the subject of a relatively large-scale Phase 1/2 clinical trial (LuMI-ERE, NCT04939610) that will evaluate the safety and efficacy of targeted FAP radiotherapy using this agent. The development of small molecule peptides that show improved engagement and tumor retention indicate that FAP is a potentially addressable target for radioligand therapy.

IMPROVING DELIVERY

Two fundamental elements are required in addition to selective uptake in tumor tissue to consider a new class of targeting ligands as potential radiotherapeutics. These characteristics are significant absolute uptake and prolonged retention in tumor. The former ensuring a significant quantity of the total radioactive drug administered is taken up by tumor cells and the latter ensures sufficient time for the deposition of energy to destroy tumor tissue. Several studies suggest that albumin-binding moieties such as long-chain fatty acids,⁴⁷ 4-(p-iodophenyl) alkanolic acid derivatives,⁴⁸ as well as ibuprofen derivatives⁴⁹ conjugated to various compounds can be used to successfully modulate plasma clearance rates in a predictable manner.

This approach to pharmacokinetic modulation has been applied recently to radiopharmaceuticals and has been shown in some cases to lead to improvements in peak tumor uptake as well as retention, resulting in an improved therapeutic efficacy.^{50–52}

This approach has recently been applied to the development of FAP ligands.⁵³ RPS-309 is a high-affinity FAP α inhibitor with prolonged plasma residence. It is a trifunctional theranostic ligand that targets FAP- α and also reversibly binds albumin and theranostic radiometals.⁵⁴ Indeed, [¹⁷⁷Lu]Lu-RPS-309 demonstrated a prolonged circulation time through the albumin-binding group as well as high affinity and retention in liposarcoma SW872 tumor-xenografted mice up to 24 hours postinjection. The multifunctional RPS-309 could be a useful tool for the study of the relationship between FAPI structure and substrate activity in the future. Others have followed suit by creating Evans Blue conjugates and other 4-(p-iodophenyl) alkanolic acid derivatives.⁵⁵ This approach requires further study as to how it may lead to improved AFP ligands with therapeutic potential.

SUMMARY

The serine protease seprase (surface expressed protease) or FAP has emerged as an attractive therapeutic target in cancer because of its upregulation in several tumor types and relative rarity in healthy tissue. Because FAP is critical to the initiation of metastatic growth, its expression will serve as a molecular marker to detect tumors at an earlier stage of development compared with currently available methods. The design of high-affinity small-molecule FAP inhibitor will allow for noninvasive imaging of activated fibroblasts in patients with cancer. Such compounds are also likely to find broad utility for imaging of various fibrogenic disorders.

The unique expression of FAP in CAFs provides the possibility of targeting a variety of FAP-positive tumors using targeted radiotherapy. Although this area of development is not as advanced as the imaging applications of FAP, the potential to target tumor stroma may have broad application.

CLINICAL CARE POINTS

- FAP imaging may have potential for assessing diseases where activated fibroblasts are known to be involved. Such diseases include myocardial remodeling, fibrotic diseases such as liver fibrosis, pulmonary fibrosis, and kidney fibrosis and cancer detection and treatment monitoring.

DISCLOSURE

Dr S.G. DiMagno and Dr J.W. Babich both are inventors on FAP-related technologies and hold equity in Ratio Therapeutics, Inc.

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