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Review

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# FAP Inhibitors as Novel Small Molecules for Cancer Imaging using Radionuclide

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**ABSTRACT**

Tumors are encircled by various non-cancerous cell types in the extracellular matrix, including fibroblasts, endothelial cells, immune cells, and cytokines. Fibroblasts are the most critical cells in the tumor stroma and play an important role in tumor development, which has been highlighted in some epithelial cancers. Many studies have shown a tight connection between cancerous cells and fibroblasts in the last decade. Regulatory factors secreted into the tumor environment by special fibroblast cells, cancer-associated fibroblasts (CAFs), play an important role in tumor and vessel development, metastasis, and therapy resistance. This review addresses the development of FAP inhibitors, emphasizing the first, second, and latest generations. First-generation inhibitors exhibit low selectivity and chemical stability, encouraging researchers to develop new scaffolds based on preclinical and clinical data. Second-generation enzymes such as UAMC-1110 demonstrated enhanced FAP binding and better selectivity. Targeted treatment and diagnostic imaging have become possible by further developing radionuclide-labeled fibroblast activation protein inhibitors (FAPIs). Although all three FAPIs (01, 02, and 04) showed excellent preclinical and clinical findings. The final optimization of these FAPI scaffolds resulted in FAPI-46 with the highest tumor-to-background ratio and better binding affinity.

**Key Words:** Fibroblast activation protein, Cancer, Cancer-associated fibroblasts, FAP inhibitors, Diagnostic imaging.

## Introduction

Tumor formation, growth, and development are mainly influenced by two important factors: cell genetic modifications and tumor microenvironment (TME) elemental modifications via inverse and active crosstalk with cancerous cells (1). Immunotherapy's growing involvement in studies has drawn more attention to the TME and its function. The important and various components of the TME include cancer-associated fibroblasts (CAFs), extracellular matrix (ECM), various types of immune cells, and blood vessels. As tumor cells mainly modify and shape the TME and manage them in line with their goals, disruption in treatment migration to other organs (2), it seems better to target the whole TME rather than the specific parts of the TME (2, 3). Fibroblasts are an extremely dominant element in the rather complicated environment of the tumor stroma, and several studies have highlighted their significance. Fibroblasts in direct contact with cancer cells have different

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names, including cancer-associated fibroblasts (CAFs), tumor-associated fibroblasts (TAFs), and mesenchymal stem cells (MSCs) (4). Due to the rarity of normal biomarkers in CAFs, it is difficult to determine their exact source; however, most scientists accept their derivation from activated fibroblasts, while fibroblasts have another origin from mesenchymal cells. Various studies have demonstrated other origins of CAFs, such as mesenchymal stem cells (MSCs) and epithelial cells (5). Fibroblast activation protein (FAP), α-smooth muscle actin, and platelet-derived growth factor receptor-β are among the most important biomarkers of CAFs (2, 6).

## Fibroblast activation protein

FAP is a cell membrane protein expressed by a broad range of fibroblasts, such as epithelial cancers and mesenchymal fibroblasts in 1986. However, because it is unavailable in almost all normal cells, it was named "fibroblast activation protein". FAP is a transmembrane serine protease, with a molecular weight of 97 kDa. It is a broad prolyl peptidase family (DPP8, DPP9, and prolyl carboxypeptidase) with a 70% amino acid sequence match to DPPIV (7-9). FAP consists of 760 amino acid sequences, with only six in the intracellular region and 20 in the transmembrane segment. The majority of its amino acid sequences are located in the extracellular domain, which contains a catalytic region and -propeller loop (10, 11). Because FAP and DPPIV have such structural similarities, many efforts have been made to determine the most important and effective differences between these two enzymes. Both enzymes' dipeptidyl peptidase activities depend on the three amino acids Glu (position 205 and 206) and Tyrosine (position 662). Finally, in 2007, Aertgeerts et al. discovered the most important amino acids responsible for their difference in activity and found that FAP contains Ala (657), while DPPIV has Asp (663) (7, 12, 13).

## FAP in Diseases

Numerous human diseases, notably arthritis, fibrosis, autoimmune diseases, atherosclerosis, metabolic disorders, and mainly cancer, are associated with FAP. In most cases, it is also responsible for the continuation of the disease (14, 15). Expression of FAP is normally low and unnoticeable in healthy tissues. Still, it is overexpressed in at least 90% of cancerous tumors, especially in breast, colorectal, pancreatic, lung, bladder, and ovarian cancers. FAP plays a role in these cancers as a significant marker in cancer-associated fibroblasts (CAFs). FAP's important function on cancer cells is classified into two distinct categories: pro-tumorigenic and anti-tumorigenic. Various mechanisms have been demonstrated to describe the effect of FAP on tumor growth, the most common of which are mentioned below.

1. Proliferation, Migration, and Invasion: influence of FAP on three important processes, proliferation, migration, and invasion, is considered the main influence of FAP. The endothelial invasion of cells, melanoma cells, and ovarian cancer cell lines through FAP has been previously reported (16, 17).
2. Epithelial-to-Mesenchymal Transition: The process of converting malignant epithelial cells into mesenchymal cells is known as epithelial-to-mesenchymal transition (EMT). This shift allows for enhanced migratory and invasive abilities, which are critical for metastasis (18).
3. Immunological Regulation/Modulation: The tumor microenvironment is an ideal location that easily accelerates the transformation of normal fibroblasts into CAFs. The regulatory power of CAFs in immune cell recruitment and function has attracted much attention. CAFs are also involved in macrophage recruitment to the tissue stroma. Following recruitment, CAFs continue their impact by supporting macrophage differentiation into M2-like macrophages, which exhibit intense immunosuppression (19, 20).
4. Support of cancer stem cells: As CAFs have a great deal of phenotypic variation and functional diversity they play an important role in treatment failure. Certain types of CAFs can restore cancer stems by promoting and proliferating cancer stem cells (CSCs) and/or encouraging the formation of CSCs from differentiated cancer cells. General control is accomplished by producing proteins and exosomes, which activate various CSC-related signals (21).

## FAP inhibitors

Although our knowledge of the details of the function of FAP in cancer cells is not comprehensive, because of its therapeutic importance, numerous attempts have been made to apply FAP biology in clinical trials. Among the reported methods, using small molecules has received special attention (22).

***Boronic Acid-Based FAP Inhibitor***

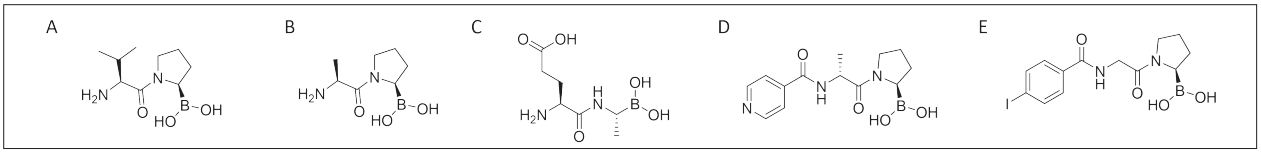

**Figure 1.** First-generation FAP inhibitors with a boronic acid warhead (A: Val-boroPro, B: Ala-boroPro, C: Glu-boroAlaB, D: ARI-3099, E: MIP-1232).

From 2006 to 2013, boronic acid organic derivatives were among the first groups studied as FAP inhibitors (Figure 1). Although some show interesting pharmacodynamic properties, they generally show little stability and low selectivity between FAP and related enzymes (23-27). Talabostat is one of the first small drugs to block the action of DPPIV and FAP dipeptidyl peptidases. Although there is no clarity regarding the mechanism of its action, it continues to be investigated in clinical experiments. Despite promising preclinical data, numerous serious effects of Talabostat have been reported, the most dangerous being related to the release of cytokines and the most common adverse event being edema (28, 29). Since Talabostat did not show sufficient selectivity for FAP, further investigation was stopped (30).

### *Xanthine core Inhibitor*

The Potent xanthine-based FAP inhibitors were developed by Veken et al. using Linagliptin as a template in 2014. The best one was selected for the structure-activity relationship study to increase the FAP-binding potency of the Linagliptin analogs. By detailed analysis of the effect of the substitution on each xanthine's different positions, the first selective non-peptide-based FAP inhibitor was developed with micromolar affinity for FAP(31).

### *Quinolinoyl core scaffold*

In a parallel study, the same group discovered the potency of the N-(4-quinolinoyl)-glycyl-(2-cyanopyrrolidine) core as one of the most promising molecules to date (UAMC1110 or Compound 60), which is considered an important lead in structure-based ligand design. This molecule showed two expected properties: low nanomolar FAP binding affinity and high FAP selectivity over DPPs/PREP. The excellent FAP-selectivity of UAMC1110 and its favorable pharmacokinetic properties make it an attractive candidate for imaging and therapy. UAMC1110 is still being studied as a potential core structure in disorders identified by FAP expression (Figure 2) (32-34).

## Fibroblast activation protein inhibitors (FAPIs): FAPI-01, 02, 04, 13, 46, and 74

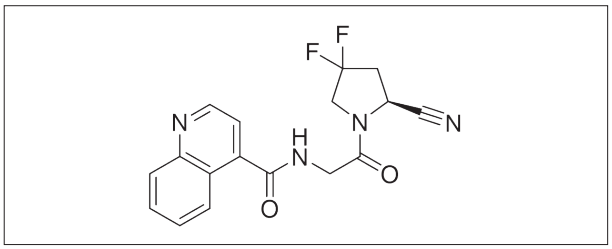

**Figure 2.** Structure of UAMC-1110.

In 2018, Loktev et al. from the Heidelberg University Hospital developed a series of quinoline-based fibroblast activation protein inhibitors (FAPIs) (34-36). The first developed FAPI was halogenated FAPI-01, and the enzymatic degradation of $^{125}$I-FAPI-01 convinced them to develop a new unhalogenated trackable core. Using a short linker, they designed FAPI-02, in which the FAP-binding motif was linked to a DOTA chelator. The above modification enables FAPI-02 to be easily employed as a diagnostic or therapeutic agent. Furthermore, it has a higher affinity for human FAP than for FAPI-01 (35-37). In both cancer-bearing animal models and human patients, $^{68}$Ga-FAPI-02 PET images demonstrated excellent tumor uptake and low noise from healthy organs (Figure 3) (35).

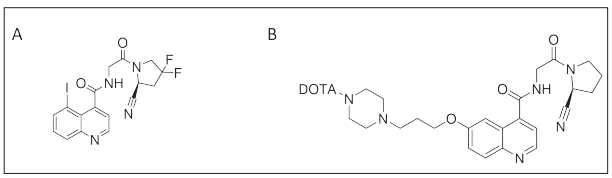

**Figure 3.** Structure of FAPI-01 and FAPI-02 (A: FAPI-01, B: FAPI-02).

A series of potential molecules based on FAPI-02 has been developed by Haberkon et al. to improve absorption and retention inside tumor cells, which led to FAPI-04 being the most effective tracer among 13 newly designed FAPIs for PET/CT imaging. FAPI-04, as the posterior version of FAPI-02, exhibits rapid penetration into FAP-expressing tumor cells followed by fast body release, resulting in similar lesion-to-organ ratios with rapid uptake at tumor spots (10 min after injection). Additionally, its effective tumor uptake was 100% greater after 24 hours compared with FAPI-02, which is extremely useful for the theranostic use of the tracer. In addition, FAPI-04 demonstrated superior stability in human blood compared to FAPI-02, more affinity for FAP than

CD26, and a slower elimination rate *in-vitro*. *In-vivo* mice study shows greater standardized uptake values, which led to a bigger area under the curve, as estimated from biological distribution tests. Subsequently, PET/CT scans with $^{68}$Ga-FAPI-04 in two women with metastatic breast tumors showed significant tracer absorption. Also, using $^{90}$Y-FAPI-04 as a therapeutic agent significantly decreased pain in female patients (36). Additionally, the $^{68}$Ga-FAPI-04 was studied in a group of 80 participants with 28 different types of malignancies. It demonstrates strong tracer absorption and high-quality images in various common malignancies (38). FAPI-13, as one of the attractive candidates, was examined more carefully due to its similar pharmacokinetic properties to FAPI-04, ultimately because of the better tumor-to-background ratio FAPI-04 was chosen (Figure 4).

The same group of scientists then tried to improve FAPI-04 pharmacokinetic characteristics and its tumor-to-background properties and selected FAPI-46 as the best one from a collection of 15 newly developed FAPIs (37) (Figure 4). Radioligand adsorption was evaluated in normal human HT-1080 FAP-expressing cells to assess the FAP-binding potency of the radiotracers, and all studies were performed in parallel with FAPI-04. All new structures showed significant and selective affinities for human FAP compared to FAPI-04 (39) (Figure 5).

Although all $^{68}$Ga-FAPIs 2, 4, and 46 have been clearly shown to be feasible PET tracers, the very short half-life of $^{68}$Ga (half-life: 68 min) causes production and distribution difficulties. If the goal is the large-scale production of $^{18}$F with a half-life of 110 min, it would overcome these limita-

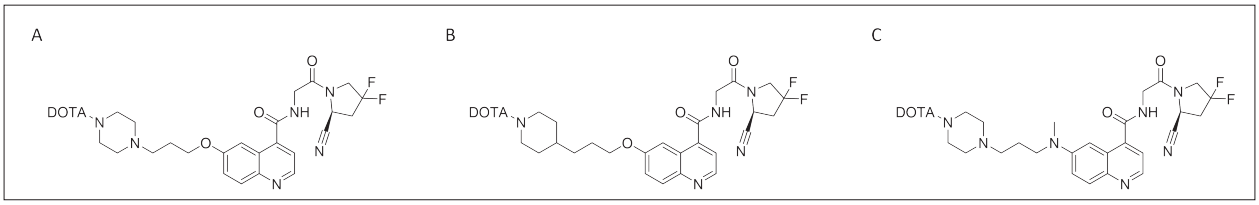


**Figure 4.** Structure of FAPI-04, 13, and 46 (A: FAPI-04, B: FAPI-13, C: FAPI-46).

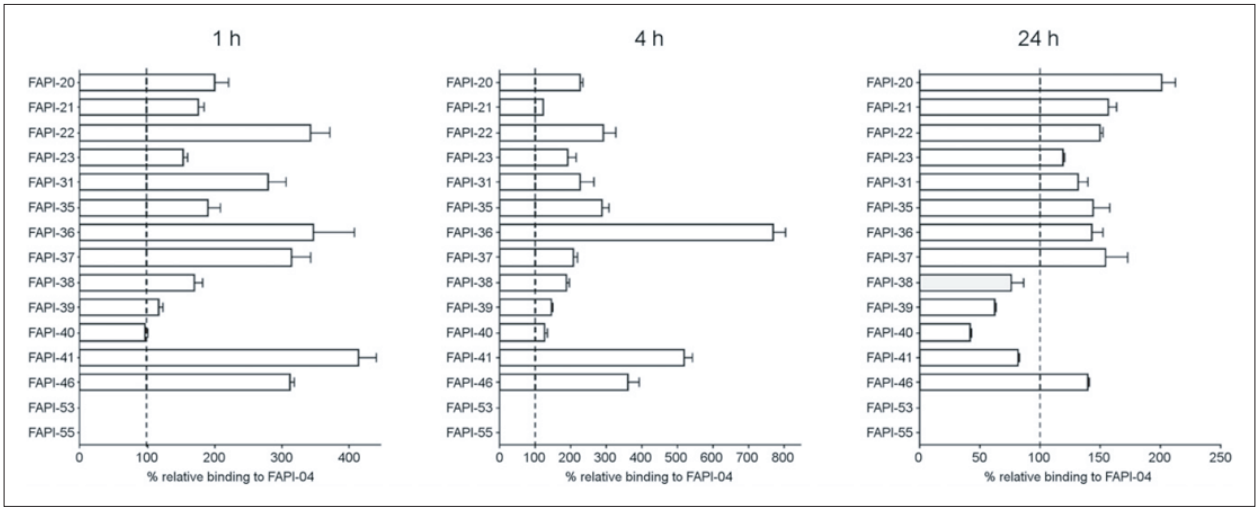


**Figure 5.** Relative binding affinity rates of FAPI derivatives compared with FAPI-04 (set to 100%) using FAP-expressing HT-1080 cells (n=3). This research was originally published in *JNM*. Anastasia Loktev et al. Development of Fibroblast Activation Protein-Targeted Radiotracers with Improved Tumor Retention. *J Nucl Med*. 2019; 60:1421-1429. © SNMMI.

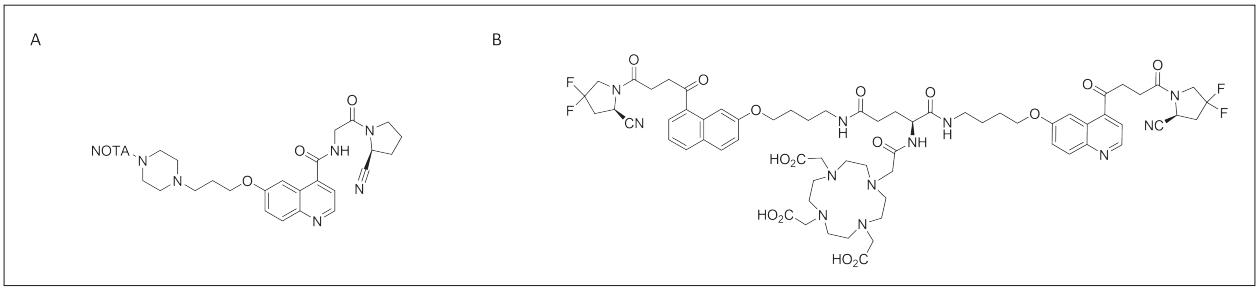


**Figure 6.** Structures of FAPI-74 and DOTA3A.Glu.(FAPi)$_2$ (A: FAPI-74, B: DOTA3A.Glu.(FAPi)$_2$).

tions in larger quantities. To address this problem, the Giesel group reported NOTA as an effective $^{18}$F-AlF/$^{68}$Ga chelator (FAPI-74) in 2021 (Figure 6A). They designed *in-vivo* experiments to understand and verify the mechanisms of $^{18}$F-FAPI-74 and $^{68}$Ga-FAPI-74 labeled tracers. Additionally, the NOTA conjugated ligand demonstrates excellent image contrast in PET/CT studies of various tumors (40).

## Later-generation and dimerized FAPIs strategy

Recent reports have utilized various possibilities to identify new generations of radiotracers. The use of peptide- and peptide-based ligands as FAP-targeting cores has shown promising results. Compared to other tracers, they mainly display long-term retention in malignant tissues (41, 42). Another interesting approach is to take advantage of the dimeric structure to discover new FAPIs, which have already been proven to increase accumulation and improve tumor retention time in specific targets, such as prostate-specific membrane antigens. This led to the design and synthesis of the first dimeric structure of DOTAGA.(SA.-FAPi)$_2$ (43). Given the positive results for the first homodimeric structure, numerous dimeric FAPI-based radiopharmaceuticals have recently been reported (44-46). Recent studies have verified that the homodimeric idea is a successful approach for targeting FAP and prolonging tumor retention. Recently, two new compounds were named DO3A.Glu.(FAPi)$_2$ and DOTAGA.Glu.(FAPi)$_2$, and tested for radiolabeling enhancements, notably for potential use in targeted alpha therapy (TAT) (47) (Figure 6B).

## Conclusion

In the last decade, FAP, one of the most abundant available biomarkers for cancer detection, has attracted the attention of researchers. In recent years, efforts to develop novel FAPIs with better pharmacodynamic characteristics, high affinity, and higher selectivity have led to the development of these radiopharmaceuticals. Although the research rate has been incredible since 2017, and the second generation has shown tremendous promise in diagnosis, tumor retention is still unacceptable. The biologically active half-life of the radiopharmaceuticals should be comparable to the actual half-life of the radionuclide for successful and ideal radiotherapy. Recently, dimerized third-generation FAPIs have attracted a lot of attention, and future research directions include improving the pharmacokinetic properties of new FAPI tracers through chemical modifications.

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