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EDITORIAL

Fibroblast activation protein (FAP)-targeted radionuclide therapy: which ligand is the best?

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Cancer ranks as a leading cause of morbidity and mortality worldwide despite early detection and management advancements made over the last few decades. According to Global Cancer Statistics, an estimated 19.3 million new cancer cases and almost 10.0 million cancer deaths occurred worldwide in the year 2020 alone [1]. While conventional diagnostic and therapeutic approaches have predominantly focused on tumor cells, it is increasingly recognized that the tumor stroma, a critical component of the tumor microenvironment (TME), plays a vital role in cancer development and progression [2]. The tumor stroma is composed of all non-malignant components in the tumor tissue, including cancer-associated fibroblasts (CAFs), the extracellular matrix (ECM), various types of immune cells, and tangled blood vessels [2]. Among them, CAFs are the most important drivers of stromal interactions which can lead to tissue remodeling, tumorigenesis, tumor stiffness, disease progression, metastasis, modulating the immune response, and treatment resistance formation [2].

A key tool in the pro-tumorigenic role of CAFs is the fibroblast activation protein (FAP), which is a type II transmembrane serine protease that cleaves peptide hormones [3]. Generally, FAP is overexpressed on the CAFs of over 90% of epithelial tumors such as breast, colorectal, head and neck, lung, ovarian, and pancreatic adenocarcinomas [2, 3]. Owing to limited FAP expression in normal tissues, it has been identified as a "pan-tumoral" target for the molecular imaging and targeted therapy of cancer [4]. Initially, few anti-FAP antibodies were proposed to arrest tumor growth in preclinical settings [5]. However, despite their initial promising results, anti-FAP antibodies have demonstrated limited clinical response towards tumor therapy in human patients [6]. Subsequently, several FAP targeting radiolabeled small molecules have been designed and developed for use in nuclear medicine, which have circumvented the limitations in the use of anti-FAP antibodies [3]. SPECT/PET imaging using these radiopharmaceuticals showed unprecedented tumor-to-organ selectivity in several hundred cancer patients. As a result, this class of radiopharmaceuticals has recently been named as "potential novel molecule(s) of the century" [7].

Based on extensive studies, a series of quinoline-based FAP inhibitors (FAPIs) was synthesized by the University Hospital Heidelberg group [3]. While the potential of these radiolabeled FAPIs as SPECT/PET imaging agents is incontestable, their suitability for therapy is impaired by their short tumor retention leading to suboptimal radiation doses to the tumor [2, 3, 8, 9]. For FAP-targeted radionuclide therapy, an evolving synthetic approach is to directly modify the molecular structure of FAPI ligands to enhance tumor uptake and prolong retention while preferably minimizing the accumulation in non-target tissues. In the past, several molecular modification strategies have been developed to attain sustained accumulation of radiolabeled FAPI ligands in tumor leading to success of the treatment [2]. The first approach is multimerization of high-affinity FAPI ligands to enhance residence time in FAP-positive tumors [10, 11], such as dimeric FAPI ligands that offer better chances of rebinding to their target, with slower off-rates than are seen with their monovalent counterparts [9–11]. Another promising strategy is to prolong the blood circulation of the radioligand by introduction of albumin binder moieties for improving the tumor uptake and retention of radiopharmaceuticals [12, 13]. Alternatively, cyclic peptides such as FAP-2286 can be synthesized which are known to have more favorable biological properties over linear counterparts, including

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greater binding affinity and selectivity due to their conformational rigidity and increased plasma stability [14]. These engineered FAPI ligands are currently being investigated in preclinical and clinical settings as theranostic agents. Nevertheless, a fair comparison among the different structural designs is essential to arrive at the best choice for FAP-targeted radiotherapeutics which could routinely be used in nuclear medicine clinics.

In a recent issue of the *European Journal of Nuclear Medicine and Molecular Imaging,* Millul et al. have systematically compared the representative FAPI ligands designed based on each of the aforementioned strategies in preclinical settings with an aim to identify the best choice for tumor therapy [15]. Monomeric FAPI-46 was used as the reference small molecule. Specifically, head-to-head comparison of monomeric FAPI-46 versus (a) its dimer (i.e., FAPI-46-F1D), (b) two albumin binding conjugates, FAPI-46-Ibu (Ibu: ibuprofen) and FAPI-46-EB (EB: Evans Blue), and (c) cyclic peptide FAP-2286 was performed (Fig. 1). Molecular modification of FAPI-46 was achieved by coupling the FAP-binding moiety ((S)-N-(2-(2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)-6-(methyl(3-(piperazin-1-yl)propyl)amino)quinoline-4-carboxamide) to aspartic acid. The free carboxylic acid in this bioconjugate was used to add Evans Blue to generate FAPI-46-EB, or for dimerization of the binding moiety to obtain FAPI-46-F1D. The chelator, DOTA, was conjugated to the N-terminal of these molecules. On the other hand, solid-phase synthesis approach was used to get FAPI-46-Ibu from beta-diamino propionic acid which was conjugated to DOTA first and then to the FAP-binding moiety. The peptide FAP-2286 was also synthesized by solid-phase peptide synthesis method. The

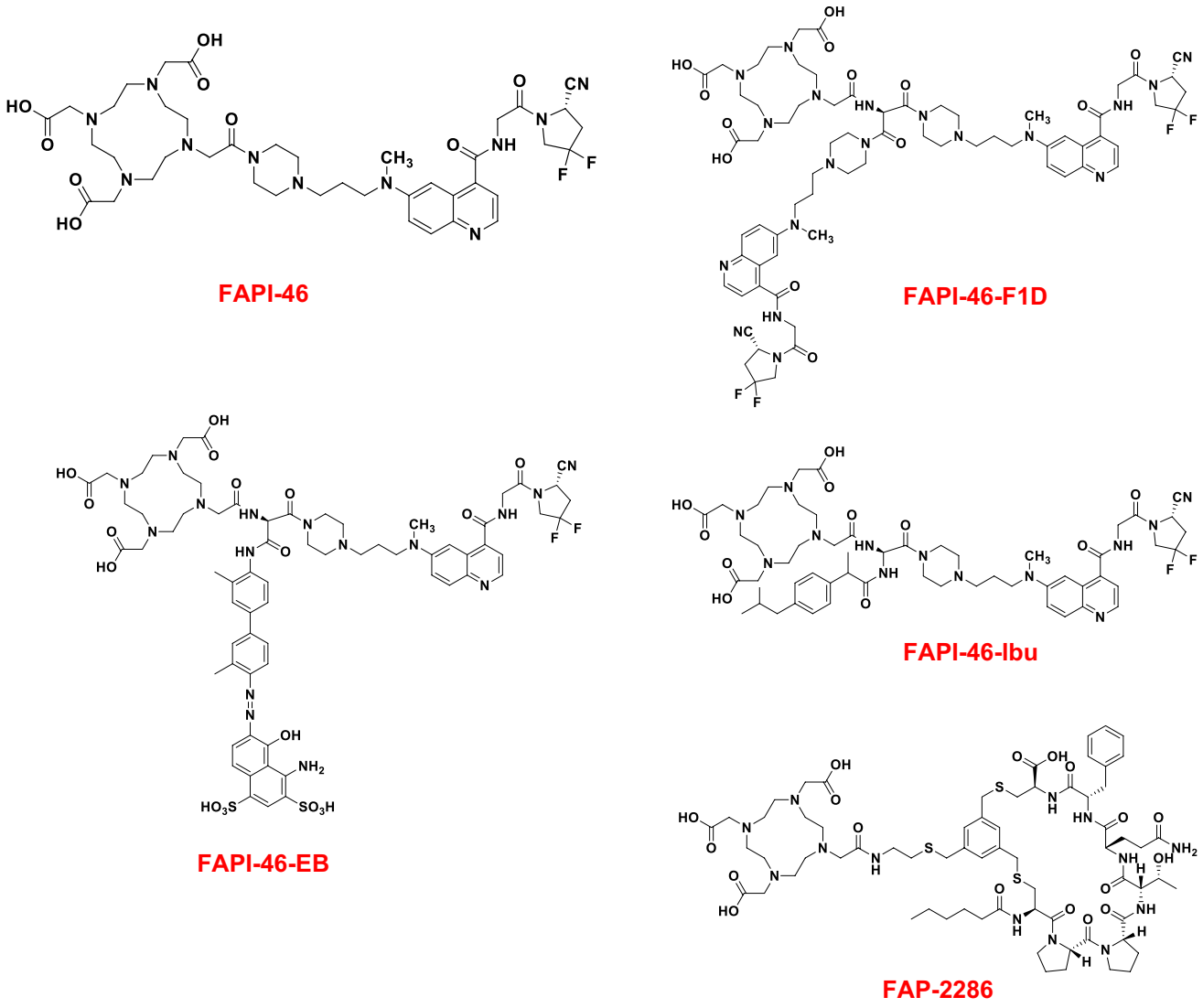

Fig. 1 Chemical structures of several FAPI-based agents described in this editorial

synthesized molecules were radiolabeled with ^{177}Lu with high yield and decent radiochemical purity. Lipophilicity of the radiolabeled agents was assessed by determining the distribution coefficient (log $D_{(pH\ 7.4)}$) values in 1-octanol/PBS system. The reference agent, [^{177}Lu]Lu-FAPI-46, was quite hydrophilic (log $D = -2.99 \pm 0.04$), and its cyclic peptide counterpart [^{177}Lu]Lu-FAP-2286 was also found to retain similar hydrophilicity (log $D = -3.43 \pm 0.17$). As expected, dimerization presented lipophilic features (log $D = -2.28 \pm 0.06$ for [^{177}Lu]Lu-FAPI-46-F1D) similar to the conjugation of Evans Blue (log $D = -2.65 \pm 0.07$ for [^{177}Lu]Lu-FAPI-46-EB). Conjugation of ibuprofen increased the lipophilicity significantly (log $D = -0.63 \pm 0.13$ for [^{177}Lu]Lu-FAPI-46-Ibu). The variation in lipophilicity modulates the routes of uptake and clearance pattern, thereby affecting the safety and efficacy of these radiopharmaceuticals.

In vitro cell binding assays were performed with different ^{177}Lu-labeled FAPI ligands in cell lines with low (HT-1080.hFAP) and high (HEK-293.hFAP) human fibroblast activation protein (hFAP) expression. In this study, radiolabeled FAPI-46-F1D ($IC_{50} = 157.8 \pm 14.5$ pM) and FAPI-46-Ibu ($IC_{50} = 39.4 \pm 16.1$ pM) showed enhanced inhibitory activity, while FAPI-46-EB ($IC_{50} = 634.3 \pm 102.3$ pM) and FAP-2286 ($IC_{50} = 247.6 \pm 71.1$ pM) showed reduced or very similar inhibitory activity compared to FAPI-46 ($IC_{50} = 247.0 \pm 17$ pM). In addition to inhibitory activity, variations were observed in the cellular distribution of the ^{177}Lu-labeled FAPI ligands. In fact, all [^{177}Lu]Lu-FAPI-46-based radioligands were near completely internalized, while [^{177}Lu]Lu-FAP-2286 remained mainly on the cell surface. Nevertheless, the high affinity of all these ligands towards hFAP as evident from the IC_{50} values allowed a reasonable assessment in vivo towards FAP-targeting.

In vivo SPECT/CT imaging and biodistribution studies were performed in HT-1080.hFAP and HEK-293.hFAP xenografts. Concentrating on the first approach of dimerization, it was demonstrated that the uptake of [^{177}Lu]Lu-FAPI-46-F1D and [^{177}Lu]Lu-FAPI-46 were independent of the tumor model and the dimer presented a higher and more persistent accumulation in the tumor compared to the monomer. Obviously, dimerization doubled the radiation dose delivered to the tumor. But in this process, doses to the non-targeted organs, such as the blood, femur, liver, and kidneys, were also increased which indicated greater toxicity. The outcome of the second strategy of the albumin binder conjugation was heavily dependent on the albumin binder moiety of choice. High concentration of [^{177}Lu]Lu-FAPI-46-EB was found in the blood at all time points, while [^{177}Lu]Lu-FAPI-46-Ibu offered a much improved clearance pattern. Nevertheless, [^{177}Lu]Lu-FAPI-46-EB demonstrated serious issues with respect to specificity and total body radiation exposure. Broadly speaking, no clear advantage of any of these two albumin bound conjugates was observed over monomeric [^{177}Lu]Lu-FAPI-46. The third approach of using cyclic peptides as alternative to small molecules offered the best choice in tumors highly expressing FAP (HEK-293.hFAP xenografts). Not only the highest but also sustained uptake of [^{177}Lu]Lu-FAP-2286 was observed in HEK293.hFAP tumors, while the lowest uptake was seen in the healthy organs compared to other radiolabeled ligands, with the exception of the kidneys. Unexpectedly, in low FAP-expressing (HT-1080.hFAP) tumors, the uptake of [^{177}Lu]Lu-FAP-2286 was significantly lower and was in fact the lowest among all studied radioligands. But still, the tumor-to-critical-organ ratio was in favor of [^{177}Lu]Lu-FAP-2286, despite the lowest tumor uptake. The kidneys are the critical organs in the study with [^{177}Lu]Lu-FAP-2286 as the tumor-to-kidney ratios were consistently high. Nevertheless, the estimated absorbed dose of [^{177}Lu]Lu-FAP-2286 to the kidneys was in the same level as the US Food and Drug Administration (US FDA)-approved radiopharmaceutical, [^{177}Lu]Lu-DOTA-TATE [16].

Overall, this study gave indications towards effective design of FAP-targeting therapeutic radiopharmaceuticals for use in clinical context. The authors inferred that compared to the more widely used FAPI monomers, dimerization of the FAPI small molecules and the synthesis of cyclic peptides are two most potent approaches for increasing tumor retention, thereby enhancing radiation dose to the tumor for effective therapy. Undoubtedly, the first observation supports the idea of using multimers by harnessing the polyvalency effect as used earlier in the improvement of pharmacokinetics of arginylglycylaspartic acid (RGD) peptide-based radiopharmaceuticals [17]. This finding could be attributed to the simultaneous binding of multiple binding motifs with the FAP leading to stronger binding, enhanced affinity, and longer retention. Moreover, when the intra-molecular distance between two adjacent binding motifs is increased using spacers such as PEG, tumor uptake and retention could be further improved. Working on these lines, recently, Pang et al. reported the synthesis of tetrameric FAPI molecules with four repeating FAPI-46 units connected by four mini-PEG spacers [18]. The radiolabeled FAPI tetramer showed higher uptake and longer retention in the tumor than its dimeric and monomeric counterparts, which resulted in improved therapeutic ability in HT-1080.hFAP and U87MG tumor-bearing mice. However, the multimerization strategy may be a double-edged sword in the development of therapeutic radiopharmaceuticals. Though it offers improved tumor uptake and retention, a steady increase of uptake in other non-target organs, e.g., liver and kidneys, was also reported, which might result in the delivery of unnecessary radiation doses and thus affect their future clinical translation. The earlier experience of comparative evaluation of in vivo biological behavior of radiolabeled RGD monomer,

dimer, and tetramer revealed that dimers exhibited considerable tumor uptake and retention with best target/non-target ratio signifying them as the best candidates for targeted tumor therapy [19]. A similar extensive study with radiolabeled FAPI multimers is warranted to arrive at a definitive conclusion. To enhance tumor uptake and retention, bi-specific heterodimeric radiotracers such as those targeting both FAPI and integrin $\alpha_v\beta_3$ (FAPI-RGD) have also been developed [20–22]. Such radiopharmaceuticals can overcome the compromised sensitivity and specificity, attributable to tumor heterogeneity and complexity of the mono-targeting radiotracers for potential therapeutic applications.

The other promising approach of using radiolabeled cyclic peptides such as [^{177}Lu]Lu-FAP-2286 for FAP-targeted radionuclide therapy was significantly wedged by the FAP-expression levels and density, which was not the case for the FAPI-46-based radioligands. Since this behavior could not be clearly explained, more detailed in vitro and in vivo studies using different cell lines with discrete features and FAP expression levels are essential to elucidate the interactions of these structurally different radioligands with FAP. Notwithstanding, the first clinical data with [^{177}Lu]Lu-FAP-2286 showed high uptake and prolonged retention in primary and metastatic tumor lesions and was relatively well tolerated with reasonable side effects [23]. It presents clinical evidence supporting the feasibility of treating different aggressive adenocarcinomas. The choice of the radionuclide also plays a significant role in FAP-targeted radionuclide therapy [8]. Generally, β^- emitting ^{177}Lu has been employed in most of the studies because of its wider commercial availability. While the FAP-targeting radiopharmaceutical ensures uptake in the tumor stroma, the cross-fire effect of the beta-emitting radionuclides would deliver tumoricidal doses to the ECM including the cancer cells. For FAPI molecules with relatively short retention time, ^{90}Y, which has a higher energy per decay and a shorter half-life, would offer a better choice. The longer range of the β^- particles of ^{90}Y compared to ^{177}Lu would also demonstrate better therapeutic benefits in larger sized tumors. FAP-targeting α-particle–based therapy using ^{211}At and ^{225}Ac has also been proposed [8]. Though these agents might effectively kill the CAFs, they have a minimal direct effect on tumor cells. Compared with ^{177}Lu, ^{225}Ac exhibited faster therapeutic effects in tumor model with a shorter duration [24]. Combined use of α- and β-emitting radiopharmaceuticals is also proposed, wherein one carrier molecule is labeled with both emitters [8]. However, superiority over the single emitter still remains unexplored. The other budding treatment strategy is to explore optimal combination therapies (targeted radionuclide therapy with external beam radiotherapy, chemotherapy, and immunotherapy) to synergistically enhance therapeutic efficacy.

In summary, we are just beginning to comprehend the potential of FAP-targeted radionuclide therapy. Extensive research on design of newer FAPI ligands is currently underway. Despite excellent attributes, there is still a very long way to go for the best FAP-targeted radiopharmaceutical to make an impact in the clinic as significant as those for [^{177}Lu]Lu-PSMA-617 in prostate cancer and [^{177}Lu]Lu-DOTA-TATE in neuroendocrine tumors. The advances in understanding the CAF biology, synthetic organic chemistry, radiopharmacy, and dosimetry might open a newer avenue for precise and personalized cancer management.

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Declarations

Conflict of interest Weibo Cai declares conflict of interest with the following corporations: Actithera, Inc., Rad Source Technologies, Inc., Portrai, Inc., rTR Technovation Corporation, and Four Health Global Pharmaceuticals, Inc. All other authors declare no conflict of interest.

Studies with human participants or animals This article does not contain any studies with human participants or animals performed by any of the authors.

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