Albumin Binder–Conjugated Fibroblast Activation Protein Inhibitor Radiopharmaceuticals for Cancer Therapy

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ABSTRACT

Fibroblast activation protein (FAP) has become an attractive target for diagnosis and therapy, and a series of FAP inhibitor (FAPI) based radiotracers have been developed and performed excellent diagnosis outcomes in clinical applications. Yet, their fast clearance and insufficient tumor retention have hampered their further clinical applications for cancer treatment. In this study, we developed two albumin binder–conjugated FAPI radiotracers, TEFAPI-06 and TEFAPI-07. They are derived from FAPI-04, and optimized by conjugating two types of well-studied albumin binders, 4-(p-iodophenyl) butyric acid moiety (TEFAPI-06) and truncated Evans blue moiety (TEFAPI-07), to try to overcome the above limitations at the expense of prolonging the blood circulation.

Methods: TEFAPI-06 and TEFAPI-07 were synthesized and labeled with ^{68}Ga , ^{86}Y and ^{177}Lu successfully. A series of cell assays were performed to identify the binding affinity and FAP specificity in vitro. PET imaging, SPECT imaging and biodistribution study were performed to evaluate the pharmacokinetics in the pancreatic cancer patient-derived xenografts (PDX) animal models. The cancer treatment efficacy of ^{177}Lu -TEFAPI-06 and ^{177}Lu -TEFAPI-07 have been evaluated in pancreatic cancer PDX-bearing mice.

Results: The binding affinity (K_d) to FAP of ^{68}Ga -TEFAPI-06 and ^{68}Ga -TEFAPI-07 is 10.16 ± 2.56 nM and 7.81 ± 2.28 nM, respectively, which were comparable with that of ^{68}Ga -FAPI-04. Comparative PET imaging of HT-1080-FAP and HT-1080 tumor–bearing

mice and blocking study showed the FAP targeting ability in vivo of these two tracers. Compared with ^{177}Lu -FAP-04, PET imaging, SPECT imaging and biodistribution studies of TEFAP-06 and TEFAP-07 have demonstrated their remarkably enhanced tumor accumulation and retention, respectively. Notable tumor growth inhibitions of ^{177}Lu -TEFAP-06 and ^{177}Lu -TEFAP-07 have been observed, while the control groups and the group treated by ^{177}Lu -FAP-04.

Conclusion: Two albumin binder-conjugated FAP radiopharmaceuticals have been developed and evaluated in vitro and in vivo. Significantly improved tumor uptake and retention have been observed compared to the original FAP tracer. Both ^{177}Lu -TEFAP-06 and ^{177}Lu -TEFAP-07 showed remarkable growth inhibition to PDX tumors while the side effect is almost negligible, showing that they are promising for further clinical translational studies.

INTRODUCTION

Fibroblast activation protein (FAP) is overexpressed in cancer-associated fibroblasts (CAFs), which is one of the main tumor stroma components and constitutes a major proportion of cells within the tumor (1,2). Though they are not malignant, the growth factor and chemokine produced by stromal cells especially CAFs can lead to the direct stimulation of tumor cell growth, migration and progression (3). Considering the vital role in tumor survival and cancer growth, CAFs-targeted diagnosis and therapy via the biomarker FAP have become an attractive strategy for tumor treatment (4,5). FAP-targeted radiopharmaceutical therapy could deliver therapeutic radioisotopes to the CAFs. It damages the stromal cells and the neighboring tumor cells through the “crossfire” effect of the β or α -emitting radionuclides, which may achieve the augmented therapeutic efficacy (6).

Recently, a variety of quinolone-based fibroblast activation protein inhibitors (FAPI) radiopharmaceuticals have been developed and demonstrated excellent uptake in different FAP-positive tumors of cancer patients (7-9). For FAP-targeted radiotherapy, an emerging strategy is to directly modify the inhibitor structure for the purpose to enhance the tumor uptake and retention, meanwhile, make the accumulation in non-target tissues remain unchanged or decreased (10-12). A series of FAPI probes including FAPI-04, FAPI-21 and FAPI-46 have been successfully developed, their improved pharmacokinetic properties

make them promising candidates for therapeutic outcome improvement (13-16). Though this is one of the optimal ways for developing therapeutic radiopharmaceuticals, the relatively rapid washout from the tumor is still a considerable limitation. Besides, it may be challenging to achieve notable enhancement of pharmacokinetic properties by subtle structure modification.

Several studies have shown that prolonging the blood circulation of drug molecules by using albumin-binder moieties could remarkably improve the therapeutic dose (17). 4-(p-iodophenyl) butyric acid and truncated Evans blue moieties are the most widely used albumin binders. The previous studies suggest that they can enhance the tumor uptake and retention of radiopharmaceuticals, resulting in improved therapeutic efficacy (18-23). Further clinical translation studies also validate the promising of this strategy to be a platform technology for radiopharmaceutical development (24-26). Therefore, we are curious if attaching an albumin-binder moiety to the FAPI molecules would improve the FAP targeted radiotherapy efficacy at the expense of increased retention in blood.

In this study, two albumin binder–FAPI conjugates, TEFAPI-06 and TEFAPI-07, are developed by logistical fabrication of three functional components: a quinoline-based FAP inhibitor originated from FAPI-04; a chelator (i.e., DOTA group) that allows radionuclide labeling for imaging (^{68}Ga or ^{86}Y) or therapy (^{177}Lu); an albumin binder, 4-(p-iodophenyl) butyric acid moiety (TEFAPI-06) or truncated Evans blue moiety (TEFAPI-07). The

purpose of the study is to evaluate whether the modification improves tumor retention in vivo, and which albumin binder matches FAPI molecules better. A series of detailed experiments and comparisons including cell binding assay, PET imaging, biodistribution and radiotherapy study have been performed. And the results demonstrated the high FAP binding affinity and specificity, the enhanced tumor retention and the improved radiotherapy efficacy of these two albumin binders conjugated FAPI radiotracers.

MATERIALS AND METHODS

Ligands and Radionuclides

The synthesis route and chemical characterization of TEFAPI-06 and TEFAPI-07 were described in the supplemental information (supplemental Fig. 1–20). $^{68}\text{GaCl}_3$ was eluted with a solution of 0.6 M hydrochloride from the ^{68}Ge - ^{68}Ga generator (iThemba LABS, South Africa). $^{86}\text{YCl}_3$ was produced with a 14.6-MeV cyclotron, the target design follows our previous report (27) and the purification procedure follows the previous protocol (28). $^{177}\text{LuCl}_3$ in a solution of 0.1 M hydrochloride was purchased from ITG (Germany).

Radiolabeling and Stability in vitro

The radiolabeling of ^{68}Ga , ^{86}Y and ^{177}Lu is performed by incubating with 50 nmol precursor at pH=4.5-5.0 at 90 °C for 10 min. The product is purified by C18 column extraction and the radiochemical purity is determined by high performance liquid chromatography

equipped with radioactivity detector (radio-HPLC). Stability of ^{177}Lu -TEFAPI-06 and ^{177}Lu -TEFAPI-07 in saline and human serum were monitored from 2 h to 168 h by using radio-HPLC (13). More details about radiochemistry, quality control and stability assay can be found in supplemental information (Section 3).

Cell Culture and Assay

The human fibrosarcoma cell line (HT-1080) and HT-1080 cell line transfected with human FAP gene (HT-1080-FAP, from WuXi AppTec) were cultivated in Eagle's minimum essential medium containing 10% fetal bovine serum, 1% antibiotic-antimycotic, 4 $\mu\text{g/mL}$ Blasticidin S at 37 °C/5% carbon dioxide condition. For competition assays, HT-1080-FAP cells were seeded in 6-well plates and cultivated until they reached about 1.2×10^6 cells per well. The cells were incubated simultaneously with unlabeled FAPI-04, TEFAPI-06 or TEFAPI-07 (10^{-5} - 10^{-9} M) with ^{68}Ga -FAPI-04 in 1 mL fresh medium without FBS for one hour. Removed the medium and washed the cells for twice with PBS. Subsequently, the cells were lysed with 0.5 mL 1 M NaOH and washed with 0.5 mL PBS twice, collected the solution of NaOH (0.5 mL) and PBS (0.5 mL $\times$ 2) to determine the uptake counts. For saturation binding assays, HT-1080-FAP and HT-1080 cells were seeded in 24-well plates and cultivated until they reached about 2×10^5 cells per well. ^{68}Ga -FAPI-04, ^{68}Ga -TEFAPI-06 or ^{68}Ga -TEFAPI-07 diluted to the concentration 0.01-200 nM in fresh medium without FBS. The cells were incubated in the above solution for one hour and then washed twice

with PBS. The lysed cells and washing PBS were collected to be determined the counts.

Tumor-Bearing Animal Models

All animal care and experimental procedure were performed by following the animal protocols (CCME-LiuZB-2) approved by the ethics committee of Peking University, and the mice were from Beijing Vital River Laboratory Animal Technology Co., Ltd. For cell-line-derived xenograft (CDX) models, 5×10^6 HT-1080-FAP or HT-1080 cells were subcutaneously inoculated into the right shoulder of 6 weeks old female nu/nu mice. For patient-derived xenograft (PDX) model establishment, the tumor specimens were obtained from patients who underwent presurgical ^{68}Ga -FAPI-04 PET/CT imaging to confirm the tumor was FAP positive. After surgical resection, the tumor specimens were immediately placed in ready to use fresh tissue preservation solution (TM2701-100) and transported under refrigerated condition within two hours. Research protocol was approved by the Institutional Ethics Committee of Peking Union Medical College Hospital (JS-2628). Six weeks old female nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice were used to establish the PDX models. After being taken out from the preservation solution, the tumor specimens were immediately immersed in sterile phosphate buffer saline solution, and minced with scissors, then the fragments were implanted subcutaneously into the left and right shoulder of the mice which were anesthetized with isoflurane in advance. Engraftment efficiency was determined by ^{68}Ga -FAPI-04 PET/CT

imaging (Supplemental Fig. 21A). The IHC staining (Supplemental Fig. 21B) demonstrated that the pancreatic cancer PDX model used in this study was indeed FAP overexpressed.

Small Animal PET Imaging

All PET scans were performed on Mediso nanoScan® PET 122S small animal PET/CT imaging system. For the 60-min dynamic PET imaging, 29.6-37.0 MBq of ^{68}Ga -TEFAPI-06 or ^{68}Ga -TEFAPI-07 were given to healthy NOD/SCID mice through tail-vein injection. The rest PET imaging was performed on pancreatic cancer PDX tumor-bearing mice, HT-1080-FAP and HT-1080 tumor-bearing mice at the indicated time points after intravenous injection of 7.4-11.1 MBq ^{86}Y -FAPI-04, ^{86}Y -TEFAPI-06 or ^{86}Y -TEFAPI-07.

Small Animal SPECT Imaging

SPECT scans were performed on Mediso nanoSPECT/CT imaging system. The ^{177}Lu -TEFAPI-06 and ^{177}Lu -TEFAPI-07 SPECT imaging were performed on the pancreatic cancer PDX-bearing mice at the indicated time points after intravenous injection of 37 MBq ^{177}Lu -TEFAPI-06 or ^{177}Lu -TEFAPI-07, respectively.

Biodistribution Study

PDX-bearing mice were injected with 925.0 kBq ^{177}Lu -TEFAPI-06 or ^{177}Lu -TEFAPI-07 for ex biodistribution. The mice were sacrificed at 24 h and 96 h after injection, the counts of different organs were measured with γ -counter and the data was normalized to ID%/g

using the 1% of total counts.

Radiotherapy Study

^{68}Ga -FAPI-04 PET imaging was performed to evaluate the tumor volume, and the mice were treated when their average tumor volume reaches 35 mm^3 . The PDX-bearing mice (six groups, 7-9 mice/group) were treated by saline, $3.7\text{ MBq }^{177}\text{Lu}$ -FAPI-04, $1.85\text{ MBq }^{177}\text{Lu}$ -TEFAPI-06, $3.7\text{ MBq }^{177}\text{Lu}$ -TEFAPI-06, $1.85\text{ MBq }^{177}\text{Lu}$ -TEFAPI-07 and $1.85\text{ MBq }^{177}\text{Lu}$ -TEFAPI-07, respectively. The tumor volume and body weight were monitored every two or three days, were euthanized when the tumor volume reached above 1000 mm^3 . Histopathologic Staining was performed with an anti-human FAP mAb (ab207178, Abcam), and the Hematoxylin and eosin (H&E) staining were performed by following the previous study (29).

RESULTS

Radiochemistry and Stability in Vitro

The radiolabeling yield of TEFAPI-06 and TEFAPI-07 (structures as in Fig. 1A) were over 90% and the radiochemical purity were over 99% ($n>20$). The specific activity of ^{68}Ga -FAPI-04, ^{68}Ga -TEFAPI-06 and ^{68}Ga -TEFAPI-07 were $5.2\text{-}6.7\text{ GBq}/\mu\text{mol}$. The specific activity of ^{86}Y -TEFAPI-06 and ^{86}Y -TEFAPI-07 were $2.2\text{-}3.4\text{ GBq}/\mu\text{mol}$. The specific activity of ^{177}Lu -FAPI-04, ^{177}Lu -TEFAPI-06 and ^{177}Lu -TEFAPI-07 were $2.9\text{-}4.4$

GBq/ μ mol. Stability of ^{177}Lu -TEFAPI-06 and ^{177}Lu -TEFAPI-07 in saline and human serum were analyzed using radio-HPLC, as shown in Supplemental Fig. 22, the radiochemistry purity of ^{177}Lu -TEFAPI-06 and ^{177}Lu -TEFAPI-07 were both still over 90% after incubated in saline and human serum for 7 days.

Binding Assay

As shown in Fig. 1B and Supplemental Fig. 23A, the cellular uptake of ^{68}Ga -FAPI-04 can be significantly inhibited by treating with cold TEFAPI-06 and TEFAPI-07. The ligand concentration required for 50% inhibition (IC_{50}) of TEFAPI-06, TEFAPI-07 is 12.24 ± 0.65 nM and 17.02 ± 1.43 nM, respectively. The dissociation constant K_d of ^{68}Ga -TEFAPI-06 and ^{68}Ga -TEFAPI-07 were 10.16 ± 2.56 nM and 7.81 ± 2.28 nM (Fig. 1C), respectively, which is comparable to that of ^{68}Ga -FAPI-04 (1.91 ± 0.62 nM, Supplemental Fig. 23B). As shown in Fig. 1D, both of ^{68}Ga -TEFAPI-06 and ^{68}Ga -TEFAPI-07 exhibit almost negligible uptake in HT-1080 cells, but have significant uptake in HT-1080-FAP cells. We have also performed the above binding assays in 0.05% human serum albumin (20). The results are as following: the IC_{50} of TEFAPI-06 and TEFAPI-07 is 11.39 ± 1.15 nM and 27.68 ± 5.00 nM in the presence of albumin, respectively; The K_d of TEFAPI-06 and TEFAPI-07 was 4.37 ± 0.81 nM and 19.12 ± 5.54 in the absence of albumin, respectively. The IC_{50} and K_d of TEFAPI-07 are slightly impacted in the presence of albumin which may be the reason why the blood clearance is faster than TEFAPI-06.

Small Animal PET Imaging

To evaluate the in vivo pharmacokinetics of these two radiotracers, dynamic PET imaging of ^{68}Ga -TEFAPI-06 and ^{68}Ga -TEFAPI-07 were performed in healthy NOD-SCID mice. The signal in heart peaked rapidly at about 2 min after injection and then declined gradually. For ^{68}Ga -TEFAPI-06, it was decreased by $35.70 \pm 4.74\%$ from 10 to 60 min after injection, which was higher than that of ^{68}Ga -TEFAPI-07 with the value of $23.15 \pm 2.16\%$, while from 60 to 240 min after injection, the signal of heart was decreased by $31.80 \pm 1.15\%$ and $40.56 \pm 5.25\%$ for ^{68}Ga -TEFAPI-06 and ^{68}Ga -TEFAPI-07 respectively, resulting in the similar decrease proportion of these two radiotracers from 10 to 240 min with the values of $56.18 \pm 2.50\%$ and $54.28 \pm 4.98\%$ for ^{68}Ga -TEFAPI-06 and ^{68}Ga -TEFAPI-07 respectively. As shown in Fig. 2, for both ^{68}Ga -TEFAPI-06 and ^{68}Ga -TEFAPI-07, most of the radioactivity is retained in the blood circulation during the monitoring period and the uptake in other organs such as liver, spleen and kidney were lower compared with the heart and main blood vessels.

To identify the tumor-targeting ability and monitor the in vivo pharmacokinetics quantitatively over a longer period. TEFAPI-06, TEFAPI-07 and FAPI-04 were labeled with the radionuclide ^{86}Y which has a half-life 14.7 h and the PET imaging was performed using pancreatic cancer PDX-bearing mice. As shown in Fig. 3 and Supplemental Fig. 24, for both ^{86}Y -TEFAPI-06 and ^{86}Y -TEFAPI-07, tumor could be completely visible at 2 h after

injection. The tumor uptake of ^{86}Y -TEFAPI-06 peaked at 0.73 (SUV mean) at 18 h after injection and that of ^{86}Y -TEFAPI-07 peaked at 0.81 (SUV mean) at 8 h after injection. Then the tumor uptake decreased slowly but still remained high uptake until 36 h after injection with the value of 0.602 and 0.606 for ^{86}Y -TEFAPI-06 and ^{86}Y -TEFAPI-07 respectively. While the tumor uptake of ^{86}Y -FAP-04 peaked at 0.35 (SUV mean) at 0.2 h after injection, and then decreased rapidly. And the area under the curve (AUC) of TEFAPI-07 and TEFAPI-06 is 35.5 folds and 37.9 folds of the AUC of FAP-04.

To further confirm the FAP specificity in vivo of these two radiotracers, PET imaging of HT-1080-FAP and HT-1080 tumor-bearing mice were performed. As shown in Fig. 4, Supplemental Fig. 25 and Supplemental Fig. 26, the uptake of ^{86}Y -TEFAPI-06 and ^{86}Y -TEFAPI-07 in HT-1080-FAP tumors was consistently 2-6 folds higher than that of in HT-1080 tumors, respectively. Blocking study was also performed, as shown in Supplemental Fig. 27, the tumor uptake decreased at 12 h and 24 h after treated with cold TEFAPI-06 and TEFAPI-07.

Small Animal SPECT Imaging

To further explore the characterization of these two molecules, SPECT imaging was conducted in PDX tumor models in a longer time. As shown in Supplemental Fig. 28, high tumor to non-targeted tissue signal ratios were observed for both ^{177}Lu -TEFAPI-06 and ^{177}Lu -TEFAPI-07 until 144 h after injection, The blood circulation properties of these two

molecules are very similar to the previous PET study.

Biodistribution Study

To further evaluate the metabolic properties in vivo, biodistribution studies using the pancreatic cancer PDX-bearing mice were performed. As shown in Fig. 5 and Table 1, the tumor uptake of ^{177}Lu -TEFAPI-06 and ^{177}Lu -TEFAPI-07 were respectively 8.68 ± 0.73 and 7.87 ± 2.08 %ID/g at 24 h after injection, and the tumor to liver ratio were 2.91 and 2.45. The tumor uptake remained high until 96 h after injection, which was 7.33 ± 2.28 and 7.57 ± 2.68 %ID/g for ^{177}Lu -TEFAPI-06 and ^{177}Lu -TEFAPI-07, and the tumor to liver ratio increased to 4.21 and 3.28, respectively. As consistent with the results of PET and SPECT imaging, the kidney uptake of ^{177}Lu -TEFAPI-07 retained high at both 24 and 96 h after injection, with the values of 8.67 ± 2.30 and 10.16 ± 3.28 %ID/g, while the kidney uptake of ^{177}Lu -TEFAPI-06 was much lower, with the value of 2.66 ± 0.54 %ID/g for 96 h after injection. By comparison, the blood clearance of ^{177}Lu -TEFAPI-06 (24 h, 13.32 ± 1.33 %ID/g; 96 h, 2.25 ± 0.68 %ID/g) was slower than that of ^{177}Lu -TEFAPI-07 (24 h, 5.64 ± 1.50 %ID/g; 96 h, 0.51 ± 0.24 %ID/g).

Radiotherapy Study

To make the assessment of therapeutic efficacy more relevant to the clinical setting, pancreatic cancer PDX-bearing mice were used for the indicated radiotherapy study (Fig. 6A). Comparing to the group treated by saline or 3.7 MBq ^{177}Lu -FAPI-04, the groups

treated by 1.85 MBq or 3.7 MBq of ^{177}Lu -TEFAPI-06 and ^{177}Lu -TEFAPI-07 showed remarkable suppression of tumor growth (Fig. 6B). No statistical difference was observed in treatment efficacy between ^{177}Lu -TEFAPI-06 and ^{177}Lu -TEFAPI-07. This result is corroborative to the PET imaging and biodistribution study, as they have equally high uptake in the tumors. Except for the control group treated with only saline, transient weight loss was observed for all treatment groups, including ^{177}Lu -FAPI-04, but then return to the healthy level after 7 d post the initial treatment. H&E staining of main organs revealed that the side effect from the treatment of ^{177}Lu -TEFAPI-06 and ^{177}Lu -TEFAPI-07 is almost negligible (Supplemental Fig. 29).

DISCUSSION

The purpose of this study is to develop FAPI-based radiopharmaceuticals that are more effective than the existing candidates for FAP targeted radiotherapy. Two different albumin binders, 4-(p-iodophenyl) butyric acid and truncated Evans blue moieties, were chosen to be attached with FAPI-04. The resulted TEFAPI-06 and TEFAPI-07 were synthesized and radiolabeled with ^{68}Ga , ^{86}Y and ^{177}Lu . The radiolabeled TEFAPIs exhibit good stability in saline and human serum, and exhibit high FAP binding affinity in vitro. In addition, SPECT imaging and biodistribution study of ^{177}Lu -TEFAPI-06 and ^{177}Lu -TEFAPI-07 showed that notable tumor uptake can still be observed even at 6 d post the injection. Meanwhile, almost

no radioactive signal could be detected for ^{177}Lu -FAPI-04 at 24 h after injection. We also wondered whether further modifications on the structure may elongate the blood circulation, thus increase the tumor accumulation. Nevertheless, it can be a challenge to balance the treatment efficacy and the potential side effect from blood circulation.

In regards to the clearance pathway, there was no significant difference of uptake between ^{177}Lu -TEFAPI-06 and ^{177}Lu -TEFAPI-07 in tumor and main organs, except for the kidney. For TEFAPI-07, both the PET and SPECT imaging results showed the significantly higher kidney uptake than that of TEFAPI-06. Of note, imaging indicated that there is no obvious clearance of TEFAPI-07 from kidney over time, which was consistent with the results of biodistribution study. Besides, as both TEFAPI-06 and ^{177}Lu -TEFAPI-07 has relatively long blood circulation than the classical radiopharmaceuticals, the side effect may not be negligible. Therefore, a comprehensive H&E staining study of major organs have been performed, and no observable tissue damage were found (Supplemental Fig. 29).

As reported in previous studies, the radiolabeled albumin binder may target the tumor due to enhanced permeability and retention of albumin (30,31). Thus, we are curious that whether the enhanced tumor uptake and retention of ^{177}Lu -TEFAPI-06 and ^{177}Lu -TEFAPI-07 are FAP-dependent. PET imaging results of FAP-positive (HT-1080-FAP) and FAP-negative (HT-1080) tumor-bearing mice showed the much higher uptake of FAP-positive tumor than that of FAP-negative tumor, which demonstrated that the higher tumor uptake

was dependent on the FAP targeting ability in vivo. For blocking study, the tumor uptake of ^{68}Ga -FAP-04 decreased significantly when the mice were treated with cold TEFAP-06 and TEFAP-07 until 24 h after injection, which supported that the prolonged tumor retention of these two radiotracers were also mainly dependent on their excellent FAP targeting ability in vivo.

CONCLUSION

In this study, two albumin binder-conjugated FAPs, denoted as TEFAP-06 and TEFAP-07, were developed to optimize the pharmacokinetics of current FAP radiopharmaceuticals for cancer radiotherapy. Compared with ^{177}Lu -FAP-04, both ^{177}Lu -TEFAP-06 and ^{177}Lu -TEFAP-07 showed enhanced uptake and retention in tumors. The tumor accumulations are highly FAP-selective and result in remarkable growth inhibition to PDX tumors while the side effect is negligible. Their promising pharmacokinetics warrant further investigations toward a clinical translation for the treatment of FAP-positive cancers.

DISCLOSURE

M. X., P. Z., J. C. and Z. L. are consultants for Borui Biotech. J. D. and L. H. have nothing to disclose. No other potential conflicts of interest relevant to this article exist.

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KEY POINTS

QUESTION: Would albumin binder–conjugated FAPI radiopharmaceuticals have high FAP specificity and give enhanced tumor retention and improved radiotherapy efficiency?

PERTINENT FINDINGS: Albumin binder–conjugated FAPI radiopharmaceuticals exhibited notably improved tumor uptake and retention compared to the original FAPI tracers, and showed remarkable growth inhibition to PDX tumors while the side effect is negligible.

IMPLICATIONS FOR PATIENT CARE: The conjugation of albumin binder is a promising strategy to develop FAP-targeted radiopharmaceuticals for treating cancers.

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Figures

FIGURE 1. (A) Chemical structures of TEFAPI-06 and TEFAPI-07. (B) Competition assays of TEFAPI-06 and TEFAPI-07; (C) Saturation binding assays of radiolabeled TEFAPI-06 and TEFAPI-07; (D) Cellular uptake assays of ^{68}Ga -TEFAPI-06 and ^{68}Ga -TEFAPI-07 in HT-1080-FAP and HT-1080 cells.

FIGURE 2. (A) Dynamic PET imaging of ^{68}Ga -TEFAPI-06 and ^{68}Ga -TEFAPI-07 in healthy NOD/SCID mice. (B) Corresponding blood time-activity curves of ^{68}Ga -TEFAPI-06 and ^{68}Ga -TEFAPI-07.

FIGURE 3. (A) PET imaging of ^{86}Y -TEFAPI-06 and ^{86}Y -TEFAPI-07 in PDX-bearing mice; (B) The time-activity curves for tumor and major organs of ^{86}Y -TEFAPI-06 and ^{86}Y -TEFAPI-07.

FIGURE 4. (A) PET imaging of ^{86}Y -TEFAPI-06 and ^{86}Y -TEFAPI-07 in HT-1080-FAP and HT-1080 tumor bearing mice at 24 h post injection; (B) The uptake of ^{86}Y -TEFAPI-06 and ^{86}Y -TEFAPI-07 in HT-1080-FAP and HT-1080 tumors at 24 h post injection (***P < 0.001).

FIGURE 5. Biodistribution study of ^{177}Lu -TEFAPI-06 (red bars) and ^{177}Lu -TEFAPI-07 (blue bars) in pancreatic cancer PDX-bearing mice at 96 h after injection (n=5).

FIGURE 6. Treatment assay of ^{177}Lu -TEFAPI-06 and ^{177}Lu -TEFAPI-07 in pancreatic cancer PDX-bearing mice. (A) Design of therapy protocols and treatment scheme; (B) The tumor growth curve after treatment (n=7-9 mice for each group); (C) Body weight change after treatment.

TABLE 1. Biodistribution results of ^{177}Lu -TEFAPI-06 and ^{177}Lu -TEFAPI-07.

Organs	^{177}Lu -TEFAPI-06				^{177}Lu -TEFAPI-07			
	24 h		96 h		24 h		96 h	
	Mean(%ID/g)	SD(%ID/g)	Mean(%ID/g)	SD(%ID/g)	Mean(%ID/g)	SD(%ID/g)	Mean(%ID/g)	SD(%ID/g)
Blood	12.32	1.33	2.25	0.68	5.64	1.49	0.51	0.24
Pancreas	2.01	0.26	0.75	0.42	2.16	0.69	0.53	0.22
Spleen	3.00	0.66	3.66	1.22	2.69	0.87	1.88	0.34
Small Intestine	2.55	0.64	0.61	0.28	1.19	0.26	0.16	0.09
Large Intestine	1.66	0.27	0.53	0.35	1.15	0.33	0.10	0.15
Liver	2.98	0.64	1.74	0.57	3.21	0.81	2.31	0.17
Kidney	3.20	0.84	2.66	0.54	8.67	2.30	10.16	3.28
Stomach	1.75	0.17	0.54	0.21	1.20	0.45	0.29	0.14
Fat	1.86	0.90	1.55	1.17	1.82	1.18	0.48	0.25
Muscle	1.09	0.18	0.45	0.05	1.00	0.37	0.28	0.16
Bone	1.32	0.30	0.35	0.20	1.71	0.63	0.18	0.28
Lung	4.33	1.44	1.95	0.70	2.58	0.75	0.73	0.28
Heart	3.70	1.59	1.51	0.42	2.53	1.19	0.62	0.17
Brain	0.37	0.08	0.09	0.03	0.23	0.09	-0.01	0.04
Tumor	8.68	0.73	7.33	2.28	7.87	2.08	7.57	2.68

GRAPHICAL ABSTRACT

Supplemental Information For

Albumin Binder–Conjugated Fibroblast Activation Protein Inhibitor

Radiopharmaceuticals for Cancer Therapy

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1. Synthesis route of TEFAPI-06 and TEFAPI-07

1.1 The synthesis route of TEFAPI-06

1.1.1 General procedure for preparation of TEFAPI-06_A2

To a solution of TEFAPI-06_A1 (20 g, 53.64 mmol, 1 *eq*, HCl) in DCM (200 mL) was added tetrahydropyran-2,6-dione (9.18 g, 80.45 mmol, 1.5 *eq*), then the solution was cooled to 0 °C. Then TEA (16.28 g, 160.91 mmol, 22.40 mL, 3 *eq*) was added drop-wise to the above solution. The mixture was stirred at 20 °C for 1 h. The solution was concentrated in vacuum, TEFAPI-06_A2 (20 g, crude) was obtained as white oil. LC-MS showed TEFAPI-06_A2 was detected.

LC-MS (ESI⁺): *m/z* 451.2 (M+H)⁺.

The column used for chromatography was a Luna C18 2.0*30 mm (3 um particles). Detection methods are diode array (DAD). MS mode was positive electrospray ionization. MS range was 100-1000. Mobile phase A was 0.037% trifluoroacetic acid in water, and mobile phase B was 0.018% trifluoroacetic acid in HPLC grade acetonitrile. The gradient was 5-95% B in 2.00 min, 5% B in 0.01 min, 5-95% B (0.01-1.00 min), 95-100% B (1.00-1.80 min), 5% B in 1.81 min with a hold at 5% B for 0.19 min. The flow rate was 1.0 mL/min (0.00-1.80 min) and 1.2 mL/min (1.81-2.00 min).

1.1.2 General procedure for preparation of TEFAPI-06_A3

To a solution of TEFAPI-06_A2 (20 g, 44.39 mmol, 1 *eq*) in MeOH (200 mL) was added Pd/C (8 g, 10% purity) at 20 °C, then the mixture was stirred at 20 °C for 12 h under H₂ (15 psi). The mixture was filtered and the filtrate was concentrated in vacuum. TEFAPI-06_A3 (14 g, crude) was obtained as white oil. LC-MS showed product was detected.

LC-MS (ESI⁺): *m/z* 317.2 (M+H)⁺.

The column used for chromatography was a Luna C18 2.0*30 mm (3 um particles). Detection methods are diode array (DAD). MS mode was positive electrospray ionization. MS range was 100-1000. Mobile phase A was 0.037% trifluoroacetic acid in water, and mobile phase B was 0.018% trifluoroacetic acid in HPLC grade acetonitrile. The gradient was 5-95% B in 2.00 min, 5% B in 0.01 min, 5-95% B (0.01-1.00 min), 95-100% B (1.00-1.80 min), 5% B in 1.81 min with a hold at 5% B for 0.19 min. The flow rate was 1.0 mL/min (0.00-1.80 min) and 1.2 mL/min (1.81-2.00 min).

1.1.3 General procedure for preparation of TEFAPI-06_A4

To a solution of 4-(4-iodophenyl) butanoic acid (15 g, 51.71 mmol, 1 *eq*) in DCM (150 mL) was added 1-hydroxypyrrolidine-2,5-dione (6.55 g, 56.88 mmol, 1.1 *eq*), then the mixture was cooled to 0 °C and DCC (12.80 g, 62.05 mmol, 12.55 mL, 1.2 *eq*) was added to the above mixture. The mixture was stirred at 20 °C

for 1 h. LC-MS showed the reaction was consumed. The mixture was filtered and the filtrate was concentrated in vacuum. Obtain (2,5-dioxopyrrolidin-1-yl) 4-(4-iodophenyl)butanoate (15 g, crude) as light yellow solid. To a solution of (2,5-dioxopyrrolidin-1-yl) 4-(4-iodophenyl)butanoate (14.68 g, 37.93 mmol, 1 *eq*) in DMF (120 mL) was added TEFAPI-06_A3 (12 g, 37.93 mmol, 1 *eq*) and DIEA (14.71 g, 113.78 mmol, 19.82 mL, 3 *eq*). The mixture was stirred at 20 °C for 1 h. LC-MS showed TEFAPI-06_A4 was detected. The mixture was purified by prep-HPLC (column: Phenomenex luna C18 250*100 mm (15 um particles); mobile phase: [water (0.1%TFA)-ACN]; B%: 35%-65%, 20 min). Obtain TEFAPI-06_A4 (10 g, 16.57 mmol, 43.68% yield, 97.5% purity) as colorless oil.

LC-MS (ESI⁺): m/z 533.1 (M/2+H)⁺.

The column used for chromatography was Xbridge Shield RP18 2.1*50 mm (5 um particles). Detection methods are diode array (DAD). MS mode was positive electrospray ionization. MS range was 100-1000. Mobile phase A was 10 mM ammonium bicarbonate in water, and mobile phase B was HPLC grade acetonitrile. The gradient was 5-95% B in 2.05 min, 5% B in 0.01 min, 5-95% B (0.01-1.00 min), 95-100% B (1.00-1.80 min), 5% B in 1.81 min with a hold at 5% B for 0.24 min. The flow rate was 1.0 mL/min.

1.1.4 General procedure for preparation of TEFAPI-06_F7

To a solution of TEFAPI-06_A4 (6.14 g, 10.43 mmol, 1.1 *eq*) in DMF (100 mL) was added HCTU (7.84 g, 18.96 mmol, 2 *eq*), HOBT (640.47 mg, 4.74 mmol, 0.5 *eq*) and DIEA (3.68 g, 28.44 mmol, 4.95 mL, 3 *eq*) at 0 °C. The mixture was stirred at 25 °C for 1 h. And then the mixture was added to a mixture of TEFAPI_A10 (2.47 g, 9.48 mmol, 1 *eq*) in resin. Then the resin was bubbled with N₂ at 25 °C for 2 h. LC-MS showed starting material was consumed completely and the desired product was detected. The mixture was filtered and the resin was washed with dimethyl formamide (4*400 mL) and dichloromethane (4*400 mL). To the above resin was added a solution of dichloromethane/trifluoroacetic acid (v/v=100:1, 600 mL) to order the

crude product can be cleavage from resin. The mixture was bubbled with N₂ for 20 min and filtered. The operation was repeated for another five times. After cleavage and filter, the filtrate was adjusted to pH 7 with NaHCO₃ (aq) and partitioned between dichloromethane (200 mL*3) and water (200 mL*3). The combined organic phases were washed with citric acid (200 mL*3). Then the mixture was concentrated under high vacuum to give TEFAPI-06_F7 (6 g, 7.11 mmol, 74.99% yield, 96.77% purity) as a yellow solid.

LC-MS (ESI⁺): m/z 817.2(M+H)⁺, Rt: 2.670 min.

The gradient was 5% B in 0.40 min and 5-95% B at 0.40-3.00 min, hold on 95% B for 1.00 min, and then 95-5 %B in 0.01 min, the flow rate was 1.0 ml/min. Mobile phase A was 0.037% trifluoroacetic acid in water, mobile phase B was 0.018% trifluoroacetic acid in acetonitrile. The column used for chromatography was a Kinetex C18 50*2.1 mm column (5 um particles). Detection methods are diode array (DAD) and evaporative light scattering (ELSD) detection as well as positive electrospray ionization.

¹H NMR (400 MHz, CDCl₃)

δ = 1.30-1.40 (m, 2H), 1.43-1.51 (m, 24H), 1.91-1.93 (m, 6H), 2.16-2.21 (m, 7H), 2.56-2.60 (m, 3H), 3.07-3.11 (m, 3H), 3.47-3.49 (m, 1H), 4.48-4.50 (m, 1H), 4.57-4.60 (m, 1H), 4.76-4.79 (m, 1H), 5.86 (br s, 1H), 6.90-6.94 (m, 2H), 7.58-7.61 (m, 2H), 7.88-7.91 (m, 1H), 8.09-8.12 (m, 1H).

1.1.5 General procedure for preparation of TEFAPI-06_8

To a solution of TEFAPI-06_F7 (858.97 mg, 1.05 mmol, 1.1 eq), *O*-benzotriazol-1-yl-tetramethyluronium hexafluorophosphate (453.23 mg, 1.20 mmol, 1.25 eq), 1-hydroxybenzotriazole (167.94 mg, 1.24 mmol, 1.3

particles). Detection methods are diode array (DAD). MS mode was positive electrospray ionization. MS range was 100-2000. Mobile phase A was 10 mM ammonium bicarbonate in water, and mobile phase B was HPLC grade acetonitrile. The gradient was 5-95% B in 2.20 min. 5% B in 0.01 min, 5-95% B (0.01-1.00 min), 95%-100% B (1.00 min-1.80 min), 100-5% B (1.80-1.81 min) with a hold at 5% B for 0.39 min. The flow rate was 1.0 mL/min (0.01-2.20 min).

The prep-HPLC method: instrument: Gilson 281 semi-preparative HPLC system; mobile phase: A: TFA/H₂O = 0.075% v/v; B: ACN; column: Phenomenex Gemini-NX 150*30 mm (5 um particles); flow rate: 25 mL/min; monitor wavelength: 220&254 nm.

The LC-MS method: the gradient was 5% B in 0.40 min and 5-95% B at 0.40-3.00 min, hold on 95% B for 1.00 min, and then 95-5% B in 0.01 min, the flow rate was 1.0 ml/min. Mobile phase A was 0.05% trifluoroacetic acid in water, mobile phase B was 0.05% trifluoroacetic acid in acetonitrile. The column used for chromatography was a Kinetex C18 50*2.1 mm column (5 um particles). Detection methods are diode array (DAD) and evaporative light scattering (ELSD) detection as well as positive electrospray ionization. MS range was 100-1500.

¹H NMR (400 MHz, DMSO-*d*₆)

δ ppm 9.18-9.07 (m, 1H), 8.84 (d, *J* = 4.4 Hz, 1H), 8.49 (br s, 1H), 8.18-7.97 (m, 3H), 7.89 (br d, *J* = 2.4 Hz, 1H), 7.78 (br t, *J* = 5.8 Hz, 1H), 7.62 (br d, *J* = 8.1 Hz, 2H), 7.55 (d, *J* = 4.4 Hz, 1H), 7.46 (br dd, *J* = 2.7 Hz, 9.1 Hz, 1H), 7.00 (br d, *J* = 8.1 Hz, 2H), 5.15 (br dd, *J* = 3.3, 9.0 Hz, 1H), 4.69 (br s, 1H), 4.47-4.08 (m, 6H), 4.07-3.69 (m, 13H), 3.64-3.22 (m, 11H), 3.21-2.69 (m, 14H), 2.47 (br s, 2H), 2.33-1.90 (m, 9H), 1.87-1.18 (m, 18H).

¹H NMR (400 MHz, methanol-*d*₄)

δ ppm 8.85 (d, *J* = 4.6 Hz, 1H), 8.22-7.97 (m, 2H), 7.71-7.53 (m, 4H), 6.99 (d, *J* = 8.3 Hz, 2H), 5.15 (br d, *J* = 9.3 Hz, 1H), 4.83-4.74 (m, 4H), 4.49-4.30 (m, 6H), 4.30-4.08 (m, 4H), 3.90-3.38 (m, 17H), 3.20-2.74 (m, 16H), 2.58 (s, 2H), 2.40 (br s, 2H), 2.30 - 2.15 (m, 6H), 2.02-1.82 (m, 6H), 1.77-0.99 (m, 11H).

1.2 The synthesis route of TEFAPI-07

The column used for chromatography was a Luna C18 2.0*30 mm (3 μ m particles). Detection methods are diode array (DAD). MS mode was positive electrospray ionization. MS range was 100-1000. Mobile phase A was 0.037% trifluoroacetic acid in water, and mobile phase B was 0.018% trifluoroacetic acid in HPLC grade acetonitrile. The gradient was 5-95% B in 2.00 min, 5% B in 0.01 min, 5-95% B (0.01-1.00 min), 95-100% B (1.00 -1.80 min), 5% B in 1.81 min with a hold at 5% B for 0.19 min. The flow rate was 1.0 mL/min.

(0.00-1.80 min) and 1.2 mL/min (1.81 -2.00 min).

1.2.2 General procedure for preparation of TEFAPI-07_F3

To a solution of TEFAPI-07_F2 (3.12 g, 9.99 mmol, 1 *eq*) in ACN (40 mL) was added HCl (2 M, 14.98 mL, 3 *eq*) at 0 °C. Then cold NaNO₂ solution (2.07 g, 29.96 mmol, 3 *eq*, in 20 mL of water) was added drop-wise to the above mixture. The mixture was stirred for 30 min. This resulting mixture was added drop-wise to another glass vial containing EB_M (3.19 g, 9.35 mmol, 0.9 *eq*) and NaHCO₃ solution (3.36 g, 39.95 mmol, 1.55 mL, 4 *eq*, in 20 mL of water) at 0 °C. The reaction was stirred at 0 °C for 2 h. LC-MS showed TEFAPI-07_F2 was consumed completely and one main peak with desired mass was detected. The reaction mixture was lyophilized to give TEFAPI-07_F3 (6 g, yield 93.48%) as a purple solid. The crude product was purified by prep-HPLC (TFA condition) to give pure product as a purple solid.

LC-MS (ESI⁻): m/z 641.1 (M-H)⁻.

The column used for chromatography was Xbridge Shield RP18 2.1*50 mm (5 um particles). Detection methods are diode array (DAD). MS mode was negative electrospray ionization. MS range was 100-2000. Mobile phase A was 10 mM ammonium bicarbonate in water, and mobile phase B was HPLC grade acetonitrile. The gradient was 5-95% B in 2.20 min, 5% B in 0.01 min, 5-95% B (0.01-1.00 min), 95-100% B (1.00-1.80 min), 100-5% B (1.80 -1.81 min) with a hold at 5% B for 0.39 min. The flow rate was 1.0 mL/min (0.01-2.20 min).

1.2.3 General procedure for preparation of TEFAPI-07_F4

To a solution of TEFAPI-07_F3 (2.2 g, 3.42 mmol, 1 *eq*) in DCM (1 mL) was added TFA (6.78 g, 59.43 mmol, 4.40 mL, 17.36 *eq*). The mixture was stirred at 25 °C for 5 h. LC-MS showed 50% of TEFAPI-07_F3 was consumed and desired MS was detected. The mixture was dried. After lyophilization, the crude product TEFAPI-07_F4 (2.2 g, yield 97.88%) was obtained as a purple solid which was used to the next step without further purification.

LC-MS (ESI⁻): *m/z* 541.0 (M-H)⁻.

The column used for chromatography was Xbridge Shield RP18 2.1*50 mm (5 um particles). Detection methods are diode array (DAD). MS mode was negative electrospray ionization. MS range was 100-2000. Mobile phase A was 10 mM ammonium bicarbonate in water, and mobile phase B was HPLC grade acetonitrile. The gradient was 5-95% B in 2.20 min, 5% B in 0.01 min, 5-95% B (0.01-1.00 min), 95-100% B (1.00-1.80 min), 100-5% B (1.80-1.81 min) with a hold at 5% B for 0.39 min. The flow rate was 1.0 mL/min (0.01-2.20 min).

1.2.4 General procedure for preparation of TEFAPI-07_F5

To a solution of TEFAPI-07_F4 (2.3 g, 4.24 mmol, 990.10 uL, 1 *eq*) in THF (30 mL) was added tetrahydropyran-2,6-dione (532.03 mg, 4.66 mmol, 1.1 *eq*) and TEA (1.29 g, 12.72 mmol, 1.77 mL, 3 *eq*). The mixture was stirred at 25 °C for 12 h. LC-MS showed 42% of TEFAPI-07_F4 remaining. Several new peaks were shown on LC-MS and ~40% of desired compound was detected. The mixture was dried under reduced pressure to get a residue. The residue was purified by prep-HPLC (TFA condition). Compound TEFAPI-07_F5 (500 mg, yield 17.96%) was obtained as a purple solid.

LC-MS (ESI⁻): *m/z* 655.1 (M-H)⁻.

The column used for chromatography was Xbridge Shield RP18 2.1*50 mm (5 um particles). Detection methods are diode array (DAD). MS mode was negative electrospray ionization. MS range was 100-2000. Mobile phase A was 10 mM ammonium bicarbonate in water, and mobile phase B was HPLC grade

acetonitrile. The gradient was 5-95% B in 2.20 min, 5% B in 0.01 min, 5-95% B (0.01-1.00 min), 95-100% B (1.00-1.80min), 100-5% B (1.80-1.81 min) with a hold at 5% B for 0.39 min. The flow rate was 1.0 mL/min (0.01-2.20 min).

1.2.5 General procedure for preparation of TEFAPI-07_F6

To a solution of TEFAPI-07_F5 (500 mg, 761.40 μmol , 1 *eq*) in DMSO (5 mL) was added HOSu (96.39 mg, 837.54 μmol , 1.1 *eq*) and EDCI (145.96 mg, 761.40 μmol , 1 *eq*). The mixture was stirred at 25 $^\circ\text{C}$ for 12 h. LC-MS showed 15% of TEFAPI-07_F5 remaining and ~80% of desired compound. The reaction mixture was used into the next step in situ.

LC-MS (ESI⁺): m/z 752.0 (M-H⁺).

The column used for chromatography was Xbridge Shield RP18 2.1*50 mm (5 μm particles). Detection methods are diode array (DAD). MS mode was negative electrospray ionization. MS range was 100-2000. Mobile phase A was 10 mM ammonium bicarbonate in water, and mobile phase B was HPLC grade acetonitrile. The gradient was 5-95% B in 2.20 min, 5% B in 0.01 min, 5-95% B (0.01-1.00 min), 95-100% B (1.00-1.80min), 100-5% B (1.80-1.81 min) with a hold at 5% B for 0.39 min. The flow rate was 1.0 mL/min (0.01-2.20 min).

1.2.6 General procedure for preparation of TEFAPI-07_F7

To a solution of F6_A (163.38 mg, 663.35 μmol , 1 *eq*) in DMSO (0.5 mL) was added TEFAPI-07_F6 (500 mg, 663.35 μmol , 1 *eq*) and DIEA (171.47 mg, 1.33 mmol, 231.09 μL , 2 *eq*). The mixture was stirred at 25

°C for 12 h. LC-MS showed 7% of TEFAPI-07_F6 remaining. Several new peaks were shown on LC-MS and ~20% of desired compound was detected. The mixture was dried under reduced pressure to get a residue. The residue was purified by prep-HPLC (TFA condition) to give TEFAPI-07_F7 (200 mg, yield 34.07%) as a dark purple solid.

LC-MS (ESI⁻): m/z 883.3(M-H)⁻.

The column used for chromatography was Xbridge Shield RP18 2.1*50 mm (5 um particles). Detection methods are diode array (DAD). MS mode was negative electrospray ionization. MS range was 100-2000. Mobile phase A was 10 mM ammonium bicarbonate in water, and mobile phase B was HPLC grade acetonitrile. The gradient was 5-95% B in 2.20 min, 5% B in 0.01 min, 5-95% B (0.01-1.00 min), 95-100% B(1.00-1.80min), 100-5% B (1.80-1.81 min) with a hold at 5% B for 0.39 min. The flow rate was 1.0 mL/min (0.01-2.20 min).

1.2.7 General procedure for preparation of TEFAPI-07_8

To a solution of FAPI_C8 (100 mg, 113.00 umol, 1 eq) in DMF (1 mL) was added TEFAPI-07_F7 (67.86 mg, 113.00 umol, 1 eq, TFA), HBTU (53.57 mg, 141.25 umol, 1.25 eq), HOBt (19.85 mg, 146.90 umol, 1.3 eq) and DIEA (73.02 mg, 564.99 umol, 98.41 uL, 5 eq). The mixture was stirred at 25 °C for 12 h. LC-MS showed TEFAPI-07_F7 was consumed and desired compound was detected. The mixture was dried under reduced pressure to get a residue. The residue was purified by prep-HPLC (TFA condition, column: Phenomenex Gemini-NX C18 75*30 mm (3 um particles); mobile phase: [water (0.1% TFA)-ACN]; B%: 15-45%, 10 min). Compound TEFAPI-07_8 (30 mg, yield 19.62%) was obtained as a purple solid.

LC-MS (ESI⁺): m/z 1354.3(M+H)⁺.

The column used for chromatography was a Luna C18 2.0*30 mm, (3 μ m particles). Detection methods are diode array (DAD). MS mode was positive electrospray ionization. MS range was 100-1000. Mobile phase A was 0.037% trifluoroacetic acid in water, and mobile phase B was 0.018% trifluoroacetic acid in HPLC grade acetonitrile. The gradient was 5-95% B in 2.00 min, 5% B in 0.01 min, 5-95% B (0.01-1.00 min), 95-100% B (1.00-1.80 min), 5% B in 1.81 min with a hold at 5% B for 0.19 min. The flow rate was 1.0 mL/min (0.00-1.80 min) and 1.2 mL/min (1.81-2.00 min).

1.2.8 General procedure for preparation of TEFAPI-07_9

To a solution of TEFAPI-07_8 (30 mg, 22.17 μ mol, 1 *eq*) in ACN (1 mL) was added TFA (308.00 mg, 2.70 mmol, 0.2 mL, 121.87 *eq*). The mixture was stirred at 25 °C for 2 h. LC-MS showed reactant 1 was consumed and a main peak with desired compound was detected. The mixture was dried under reduced pressure to get a residue. The crude product TEFAPI-07_9 (30 mg, yield 98.98%) was obtained as a purple solid which was used for the next step without further purification.

1.2.9 General procedure for preparation of TEFAPI-07_10

To a solution of TEFAPI-07_9 (15 mg, 10.97 μ mol, 1 *eq*, TFA) in DMSO (1 mL) was added FAPI_D2 (8.82

mg, 13.16 μmol , 1.2 *eq*) and DIEA (7.09 mg, 54.85 μmol , 9.55 μL , 5 *eq*). The mixture was stirred at 25 °C for 12 h. LC-MS showed reactant 1 was consumed and desired compound was detected. The mixture was dried under reduced pressure to get a residue. Two additional vials were set up as described above. All three reaction mixtures were combined. The residue was purified by prep-HPLC (TFA condition, column: Phenomenex Gemini-NX 150*30 mm (5 μm particles); mobile phase: [0.1 M TEAB-ACN]; B%: 10-75%, 10 min. The product TEFAPI-07_10 (20 mg, yield 75.82%) was obtained as a purple solid.

LC-MS (ESI⁺): m/z 1847.0 (M+MeCN)⁺.

The column used for chromatography was a Luna C18 2.0*30 mm (3 μm particles). Detection methods are diode array (DAD). MS mode was positive electrospray ionization. MS range was 100-1000. Mobile phase A was 0.037% trifluoroacetic acid in water, and mobile phase B was 0.018% trifluoroacetic acid in HPLC grade acetonitrile. The gradient was 5-95% B in 2.00 min .5% B in 0.01 min, 5-95% B (0.01-1.00 min), 95-100% B (1.00 -1.80 min), 5% B in 1.81 min with a hold at 5% B for 0.19 min. The flow rate was 1.0 mL/min (0.00-1.80 min) and 1.2 mL/min (1.81-2.00 min).

1.2.10 General procedure for preparation of TEFAPI-07

To a solution of TEFAPI-07_10 (20 mg, 11.06 μmol , 1 *eq*) in DCM (3 mL) was added TFA (18.48 g, 162.07 mmol, 12.00 mL, 14651.92 *eq*). The mixture was stirred at 25 °C for 8 h. LC-MS showed reactant 1 was consumed, a main peak with desired compound was detected. The mixture was dried under reduced pressure to get a residue. The residue was purified by prep-HPLC (basic condition, column: Phenomenex Gemini-NX 150*30 mm (5 μm particles); mobile phase: [0.1 M TEAB-ACN]; B%: 10-35%, 20 min). Compound TEFAPI-07 (7.62 mg, yield 41.38%) was obtained as a purple solid.

¹H NMR (400 MHz, DMSO-*d*₆)

δ = 1.18-1.30 (m, 3H), 1.33-1.42 (m, 2H), 1.44-1.58 (m, 2H), 1.74-2.13 (m, 11H), 2.17-2.25 (m, 3H), 2.28 (s, 3H), 2.31-2.40 (m, 5H), 2.57-2.72 (m, 6H), 2.89 (br s, 22H), 4.05-4.40 (m, 7H), 4.60-4.76 (m, 1H), 5.11-5.22 (m, 1H), 6.99 (br d, $J=9.63$ Hz, 1H), 7.42-7.60 (m, 5H), 7.64 (br s, 2H), 7.88 (br d, $J=8.38$ Hz, 2H), 7.93-8.07 (m, 2H), 8.28-8.41 (m, 1H), 8.57-8.71 (m, 1H), 8.80 (br d, $J=4.13$ Hz, 1H), 9.15-9.31 (m, 1H), 9.31-9.47 (m, 1H), 15.84-16.05 (m, 1H).

1.3 The synthesis route of FAPI_C8

1.3.1 General procedure for preparation of FAPI_F2

A solution of 5-methoxyindoline-2,3-dione (20 g, 112.89 mmol, 1 eq) and KOH (69.68 g, 1.24 mol, 11 eq)

in H₂O (200 mL) was added Pyruvic acid (10.94 g, 124.18 mmol, 8.75 mL, 1.1 eq) at 25 °C. The reaction mixture was warmed to 40 °C and stirred at 40 °C for 15 hours. LC-MS showed the starting material was consumed completely. The reaction mixture was cooled to 15 °C and acidified with HCl to pH 3, the precipitated solid was collected by filtration and washed with deionized water, FAPI_F2 (24 g, 92.23 mmol, 81.70% yield, 95% purity) was obtained as grey solid.

¹H NMR: (400 MHz, DMSO-*d*₆)

δ 3.70-4.24 (m, 3H), 7.58 (dd, *J* = 9.24, 2.80 Hz, 1H), 8.15 (d, *J* = 9.30 Hz, 1H), 8.26 (d, *J* = 2.74 Hz, 1H), 8.53 (s, 1H), 13.75 (br s, 2H).

LC-MS(ESI⁺): *m/z* 248.1 (M+H)⁺.

LC-MS method: the column used for chromatography was a ZORBAX Eclipse XDB C18 2.1*30 mm (3.5 μm particles). Detection methods are diode array (DAD). MS mode was positive electrospray ionization. MS range was 100-1000. Mobile phase A was 0.037% trifluoroacetic acid in water, and mobile phase B was 0.018% trifluoroacetic acid in HPLC grade acetonitrile. The gradient was 5-95% B in 2.20 min. 5% B in 0.01 min, 5-95% B (0.01-1.00 min), 95-100% B (1.00-1.80 min), 5% B in 1.81 min with a hold at 5% B for 0.39 min. The flow rate was 1.0 mL/min.

1.3.2 General procedure for preparation of FAPI_C3

A solution of FAPI_F2 (24 g, 97.09 mmol, 1 eq) in nitrobenzene (150 mL) was stirred at 220 °C for 1.5 hours. LC-MS showed the starting material was consumed completely. The reaction mixture was cooled to 15 °C and diluted with PE (150 mL). The precipitated solid was collected by filtration and washed with PE (100 mL) to afford FAPI_C3 (16.8 g, 82.68 mmol, 85.16% yield) as grey solid, which was used to the next step without purification. The structure was confirmed by HNMR.

¹H NMR: (400 MHz DMSO-*d*₆)

δ 3.72-4.14 (m, 3H), 7.49 (dd, $J = 9.23, 2.75$ Hz, 1H), 7.93 (d, $J = 4.40$ Hz, 1H), 8.02 (d, $J = 9.17$ Hz, 1H), 8.18 (d, $J = 2.69$ Hz, 1H), 8.87 (d, $J = 4.40$ Hz, 1H), 13.76 (br s, 1H).

LC-MS(ESI⁻): m/z 202.1 (M-H)⁻.

LC-MS method: the column used for chromatography was a ZORBAX Eclipse XDB C18 2.1*30 mm (3.5 μ m particles). Detection methods are diode array (DAD). MS mode was positive electrospray ionization. MS range was 100-1000. Mobile phase A was 0.037% NH₄HCO₃ in water, and mobile phase B was HPLC grade acetonitrile. The gradient was 5-95% B in 2.20 min, 5% B in 0.01 min, 5-95% B (0.01-1.00 min), 95-100% B (1.00-1.80 min), 5% B in 1.81 min with a hold at 5% B for 0.39 min. The flow rate was 1.0 mL/min.

1.3.3 General procedure for preparation of FAPI_C4

A mixture of FAPI_C3 (40 g, 196.86 mmol, 1 *eq*) in aqueous solution of hydrogen bromide (1 L, 48% in water) was stirred at 130 °C for 12 hrs. LC-MS showed FAPI_C3 was consumed, several new peaks were shown on LC-MS and ~81.3% of desired compound was detected. The reaction mixture was basified by adding 350 ml 30% of sodium hydroxide aqueous solution dropwise to pH 6, a large amount of solid were precipitate out, then the solid was collected by filtration, the filter cake was washed with methanol and dried under vacuum to give crude product. The crude product was triturated with methanol (200 ml) and collected by filtration. FAPI_C4 (30 g, 128.93 mmol, 65.50% yield, 81.3% purity) was obtained as a brown solid.

LC-MS (reaction) (ESI⁻): m/z 377.0 (2M-H)⁻.

LC-MS method: the column used for chromatography was Xbridge Shield RP18 2.1* 50 mm (5 μ m particles). Detection methods are Diode Array detector (DAD). MS mode was negative electrospray ionization. MS range was 100-1000. Mobile phase A was 10 mM ammonium bicarbonate in water, and mobile phase B was HPLC grade acetonitrile. The gradient was 0-30% B in 4.30 min, 0% B in 0.01 min, 0-30% B (0.01-3.50 min), with a hold at 30% B for 0.3 min, 30-0% B (3.80-3.81 min), with a hold at 0% B for 0.50 min. The

flow rate was 0.8 mL/min (0.01-3.80 min) and 1.0 mL/min (3.81-4.30 min).

¹H NMR (400 MHz, DMSO-*d*₆)

δ ppm 13.66 (br s, 1H), 10.24 (s, 1H), 8.77 (d, *J* = 4.5 Hz, 1H), 8.06 (d, *J* = 2.6 Hz, 1H), 7.95 (d, *J* = 9.1 Hz, 1H), 7.84 (d, *J* = 4.4 Hz, 1H), 7.36 (dd, *J* = 2.7, 9.1 Hz, 1H).

1.3.4 General procedure for preparation of FAPI_C5B

To a stirred solution of FAPI_C4 (30 g, 158.59 mmol, 1 *eq*) in dimethyl formamide (300 mL) was added potassium carbonate (87.67 g, 634.36 mmol, 4 *eq*) and 1-bromo-3-chloro-propane (74.90 g, 475.77 mmol, 46.81 mL, 3 *eq*), then the reaction mixture was stirred at 60 °C for 12 hrs. LC-MS showed the FAPI_C4 was consumed and the product was detected with the desired MS. The reaction mixture was concentrated under reduced pressure and the residue was added water (200 mL), a large amount of solid were precipitate out, then the resulting mixture was stirred at 25 °C for 15 mins and filtered in vacuum. The crude product was used for next step directly without further purification. FAPI_C5B (51.69 g, 141.98 mmol, 89.53% yield, 94% purity) was obtained as a brown solid (Note: the product is a mixture of Cl and Br compound).

LC-MS (reaction) (ESI⁺): *m/z* 342.8 (M+H)⁺.

LC-MS method: the column used for chromatography was a Chromolith RP-18e 25-2 mm. Detection methods are diode array (DAD). MS mode was positive electrospray ionization. MS range was 100-1000. Mobile phase A was 0.04% trifluoroacetic acid in water, and mobile phase B was 0.02% trifluoroacetic acid in HPLC grade acetonitrile. The gradient was 5-95% B in 1.50 min, 5% B in 0.01 min, 5-95% B (0.01-0.70 min), 95-100% B (0.70-1.15min), 5% B in 1.16 min with a hold at 5% B for 0.34 min. The flow rate was 1.5 mL/min.

1.3.5 General procedure for preparation of FAPI_C5

To a stirred solution of FAPI_C5B (51.69 g, 151.04 mmol, 1 *eq*) in water (270 mL), tetrahydrofuran (170 mL), methanol (170 mL) was added lithium hydroxide (10.85 g, 453.13 mmol, 3 *eq*) in portions at 25 °C, and the reaction mixture was stirred at 25 °C for 12 hrs. LC-MS showed FAPI_C5B was consumed, one main peak was shown on LC-MS and ~84.47% of desired compound was detected. The reaction mixture was concentrated under reduced pressure to remove tetrahydrofuran and methanol, then the resulting mixture was acidified by adding 12 mol/L hydrochloric acid dropwise at 0 °C to pH 7, a large amount of solid were precipitate out, the residue was triturated with ethyl acetate (200 ml × 2) and collected by filtration under vacuum. FAPI_C5 (37.58 g, 130.13 mmol, 86.15% yield, 92% purity) was obtained as a brown solid (Note: the product is a mixture of Cl and Br compound).

LC-MS (reaction) (ESI⁺): m/z 266.1 (M+H)⁺.

LC-MS method: the column used for chromatography was a Chromolith RP-18e

25-2 mm. Detection methods are diode array (DAD). MS mode was positive electrospray ionization. MS range was 100-1000. Mobile phase A was 0.04% trifluoroacetic acid in water, and mobile phase B was 0.02% trifluoroacetic acid in HPLC grade acetonitrile. The gradient was 5-95% B in 1.50 min, 5% B in 0.01 min, 5-95% B (0.01-0.70 min), 95-100% B (0.70-1.15 min), 5% B in 1.16 min with a hold at 5% B for 0.34 min. The flow rate was 1.5 mL/min.

¹H NMR (400 MHz, DMSO-*d*₆)

δ ppm 2.26-2.98 (m, 2 H), 3.83-3.88 (m, 2 H), 4.22-4.25 (m, 2 H), 7.54 (dd, *J* = 9.19, 2.69 Hz, 1 H), 7.96 (d, *J* = 4.38 Hz, 1 H), 8.03-8.11 (m, 1 H), 8.19 (d, *J* = 2.63 Hz, 1 H), 8.90 (d, *J* = 4.38 Hz, 1 H).

1.3.6 General procedure for preparation of FAPI_C6

A mixture of FAPI_C5 (30 g, 112.91 mmol, 1 *eq*), tert-butyl piperazine-1-carboxylate (105.15 g, 564.56 mmol, 5 *eq*) and potassium iodide (9.37 g, 56.46 mmol, 0.5 *eq*) in *N*-methyl-pyrrolidone (300 mL) was stirred at 60 °C for 12 hrs. LC-MS showed all FAPI_C5 was consumed and the product was detected with the desired MS. The reaction mixture was cooled to room temperature and filtered. The crude product was purified by reversed-phase HPLC (0.1% FA condition). FAPI_C6 (42.96 g, 96.16 mmol, 85.16% yield, 93% purity) was obtained as yellow solid.

LC-MS (reaction) (ESI⁺): *m/z* 416.1 (M+H)⁺.

LC-MS method: the column used for chromatography was a Kinetex C18 2.1*30 mm (5 um particles). Detection methods are diode array (DAD). MS mode was positive electrospray ionization. MS range was 100-1000. Mobile phase A was 0.04% TFA in water, and mobile phase B was 0.02% TFA in HPLC grade acetonitrile. The gradient was 5-95% B in 4.30 min, 5% B in 0.01 min, 5-95% B (0.01-3.00 min), with a hold at 95% B for 0.50 min, 95-5% B (3.50-3.51 min), 5% B in 3.51 min, with a hold at 5% B for 0.79 min. The flow rate was 1.0 mL/min.

¹H NMR (400 MHz, DMSO-*d*₆)

δ ppm 8.82 (d, *J* = 4.4 Hz, 1H), 8.19-8.16 (m, 1H), 7.99 (d, *J* = 9.3 Hz, 1H), 7.85 (d, *J* = 4.4 Hz, 1H), 7.45 (dd, *J* = 2.8, 9.1 Hz, 1H), 4.15 (br t, *J* = 6.2 Hz, 2H), 2.62-2.50 (m, 6H), 2.42 (br t, *J* = 4.6 Hz, 4H), 1.99 (br d, *J* = 6.6 Hz, 2H), 1.39 (s, 9H).

1.3.7 General procedure for preparation of FAPI_C7

To a stirred solution of FAPI_C6 (12.00 g, 26.57 mmol, 92% purity, 1.02 *eq*), *O*-benzotriazol-1-yl-tetramethyluronium hexafluorophosphate (19.76 g, 52.10 mmol, 2 *eq*), 1-hydroxybenzotriazole (7.04 g, 52.10 mmol, 2 *eq*), *N,N*-diisopropylethylamine (10.10 g, 78.15 mmol, 13.61 mL, 3 *eq*) in dimethyl formamide (120 mL) was added FAPI_B6 (5.88 g, 26.05 mmol, 1.00 *eq*, HCl), and the reaction mixture was stirred at 25 °C for 12 hrs. LC-MS showed the FAPI_C6 was consumed, several new peaks were shown on LC-MS and ~50.0% of desired compound was detected. The reaction mixture was filtered. The filtrate was purified by prep-HPLC (HCl condition), column: Phenomenex luna C18 250*70 mm (15 um particles); mobile phase: [water (0.05% HCl)-ACN]; B%: 5%-35%, 22 min. FAPI_C7 (11.27 g, 17.29 mmol, 66.37% yield, 90% purity) was obtained as a yellow solid.

LC-MS (reaction) (ESI⁺): *m/z* 587.2 (M+H)⁺.

LC-MS method: the column used for chromatography was a Kinetex C18 2.1*30 mm (5 um particles). Detection methods are diode array (DAD). MS mode was positive electrospray ionization. MS range was 100-1000. Mobile phase A was 0.04% TFA in water, and mobile phase B was 0.02% TFA in HPLC grade acetonitrile. The gradient was 5-95% B in 4.30 min, 5% B in 0.01 min, 5-95% B (0.01-3.00 min), with a hold at 95% B for 0.50 min, 95-5% B (3.50-3.51 min), 5% B in 3.51 min, with a hold at 5% B for 0.79 min. The flow rate was 1.0 mL/min.

¹H NMR (400 MHz, DMSO-*d*₆)

δ ppm 1.41 (s, 9 H), 2.27-2.33 (m, 2 H), 3.27-3.29 (m, 5 H), 3.43-3.55 (m, 2 H), 3.93-4.21 (m, 10 H), 5.19

(dd, $J = 9.26, 2.75$ Hz, 1 H), 7.59 (dd, $J = 9.26, 2.63$ Hz, 1 H), 7.69 (d, $J = 4.63$ Hz, 1 H), 7.87-8.00 (m, 1 H), 8.14 (d, $J = 9.26$ Hz, 1 H), 8.95 (d, $J = 4.63$ Hz, 1 H), 9.24 (br t, $J = 5.82$ Hz, 1 H), 11.00 (br s, 1 H).

1.3.8 General procedure for preparation of FAPI_C8

To a stirred solution of FAPI_C7 (2 g, 3.41 mmol, 1 *eq*) in ethyl acetate (40 mL) was added hydrogen chloride in ethyl acetate solution (4 M, 4 mL, 4.69 *eq*), and the reaction mixture was stirred at 25 °C for 6 hrs. LC-MS showed FAPI_C7 was consumed, one main peak was shown on LC-MS and ~81.0% of desired compound was detected. The reaction mixture was concentrated under reduced pressure to remove ethyl acetate. The crude product was used for next step directly without further purification. FAPI_C8 (1.5 g, crude, HCl) was obtained as a yellow solid.

LC-MS (reaction) (ESI⁺): m/z 485.1 (M+H)⁺.

LC-MS method: the column used for chromatography was Xbridge Shield RP18 2.1*50 mm (5 um particles). Detection methods are diode array (DAD). MS mode was negative electrospray ionization. MS range was 100-1000. Mobile phase A was 10 mM ammonium bicarbonate in water, and mobile phase B was HPLC grade acetonitrile. The gradient was 5-95% B in 4.30 min, 5% B in 0.01 min, 5-95% B (0.01-3.00 min), and hold at 95% B within 0.5 min, 95-5% B (3.50-3.51 min), with a hold at 5% B for 0.79 min. The flow rate was 1.0 mL/min (0.01-4.30 min).

1.4 The synthesis route of FAPI_D2

1.4.1 General procedure for preparation of FAPI_D2

To a solution of FAPI_D1 (60 mg, 104.76 μmol , 1 *eq*) and *O*-benzotriazol-1-yl-tetramethyluronium hexafluorophosphate (47.68 mg, 125.71 μmol , 1.2 *eq*) in acetonitrile (0.6 mL) was added 1-hydroxypyrrolidine-2,5-dione (14.47 mg, 125.71 μmol , 1.2 *eq*), and the reaction mixture was stirred at 25 °C for 12 hrs. TLC (dichloromethane : acetonitrile = 10:1) showed FAPI_D1 was consumed and desired compound was detected. The reaction mixture was concentrated under reduced pressure to remove acetonitrile. The mixture was purified by prep-TLC (SiO_2 , dichloromethane: acetonitrile = 10:1). Compound FAPI_D2 (29 mg, 43.30 μmol , 41.33% yield) was obtained as a white solid. The structure was confirmed by HNMR.

^1H NMR (400 MHz, chloroform-*d*)

δ ppm 1.46 (s, 27 H), 2.03 (s, 8 H), 2.70-2.94 (m, 16 H), 3.01-3.13 (m, 4 H).

1.5. NMR Spectra and mass spectrum

Supplemental Fig 1. LC-MS analysis of TEFAPI-06_F7.

Report

Signal 1 : DAD1 E, Sig=220,4 Ref=off

Peak #	RT [min]	Height	Height %	Width [min]	Area	Area %
1	0.805	12.308	2.525	0.054	41.760	3.222
2	2.670	475.042	97.475	0.043	1254.486	96.778

Signal 2 : DAD1 D, Sig=254,4 Ref=off

Peak #	RT [min]	Height	Height %	Width [min]	Area	Area %
1	2.670	44.833	100.000	0.043	118.920	100.000

Signal 3 : ADC1 A, ELSD

Peak #	RT [min]	Height	Height %	Width [min]	Area	Area %
1	2.743	129.523	100.000	0.029	254.433	100.000

Supplemental Fig 2. MS analysis of TEFAPI-06_F7.

Supplemental Fig 3. ^1H NMR analysis of TEFAPI-06_F7.

Supplemental Fig 4. LC-MS analysis of TEFAPI-06.

Report

Signal 1 : DAD1 E, Sig=220,4 Ref=off

Peak #	RT [min]	Height	Height %	Width [min]	Area	Area %
1	1.959	802.222	100.000	0.048	2371.837	100.000

Signal 2 : DAD1 B, Sig=254,4 Ref=off

Peak #	RT [min]	Height	Height %	Width [min]	Area	Area %
1	1.959	598.614	100.000	0.048	1755.644	100.000

Signal 3 : ADC1 A, ELSD

Peak #	RT [min]	Height	Height %	Width [min]	Area	Area %
1	2.044	60.568	100.000	0.042	163.659	100.000

Supplemental Fig 5. MS analysis of TEFAPI-06.

Supplemental Fig 6. ^1H NMR (DMSO- d_6) analysis of TEFAPI-06.

Supplemental Fig 7. ^{19}F NMR (DMSO- d_6) analysis of TEFAPI-06.

Supplemental Fig 8. ^1H NMR (methanol- d_4) analysis of TEFAPI-06.

Supplemental Fig 9. ^{19}F NMR (methanol- d_4) analysis of TEFAPI-06.

Supplemental Fig 10. ^1H NMR analysis of TEFAPI-06.

Supplemental Fig 11. ^{19}F NMR analysis of TEFAPI-06.

Supplemental Fig 12. ^1H NMR analysis of FAPI_F2.

Supplemental Fig 13. ^1H NMR analysis of FAPI_C3.

Supplemental Fig 14. ^1H NMR analysis of FAPI_C4.

Supplemental Fig 15. ^1H NMR analysis of FAPI_C5.

Supplemental Fig 16. ^1H NMR analysis of FAPI_C6.

Supplemental Fig 17. ^1H NMR analysis of FAPI_C7.

Supplemental Fig 18. ^{19}F NMR analysis of FAPI_C7.

Supplemental Fig 19. MS analysis of FAPI_C8.

Supplemental Fig 20. ^1H NMR analysis of FAPI_D2.

2. ^{68}Ga -FAP-04 PET imaging and IHC staining results of pancreatic PDX tumor

Supplemental Fig 21. (A) PET imaging of four pancreatic PDX tumor bearing mice using ^{68}Ga -FAP-04. (B) IHC staining of FAP expression and HE staining in excised pancreatic PDX tumor.

3. Radiolabeling and HPLC test procedure and stability results of in vitro stability analysis

^{68}Ga -FAPI-04, ^{68}Ga -TEFAPI-06 and ^{68}Ga -TEFAPI-07 were prepared using sodium hydroxide (3 M) and sodium acetate (3 M) to adjust the $^{68}\text{GaCl}_3$ solution in 0.6 M hydrochloric acid to pH 4.5, the mixture was heated at 90 °C for 10 min to obtain the labeling product. ^{86}Y -TEFAPI-06 and ^{86}Y -TEFAPI-07 were prepared using sodium acetate (3 M) to adjust the $^{86}\text{YCl}_3$ solution in 0.1 M hydrochloric acid to pH 4.5, the labeling condition was also incubation at 90 °C for 10 min. ^{177}Lu -FAPI-04, ^{177}Lu -TEFAPI-06 and ^{177}Lu -TEFAPI-07 were prepared in sodium acetate buffer (pH 4.5-5.0, 0.2 M), the mixture was heated at 90 °C for 10 min. The pH of the labeling mixture was detected using pH-indicator strips. After heating, the reaction mixture was diluted into 5 mL saline in a syringe and purified by using a pre-conditioned Sep-Pak Light C18 cartridge (Waters) activated by ethanol (5 mL) and water (5 mL). For radiolabeled TEFAPI-06, the product was eluted with about 300 μL EtOH, and for radiolabeled TEFAPI-07, the product was eluted with about 300 μL EtOH/saline solution containing 20% saline. The elution of the radiolabeling product was diluted with saline to control the EtOH content was under 10% for injection. All the labeling products were purified by solid phase extraction and qualified by high performance liquid chromatography equipped with radioactivity detector (radio-HPLC). Stability of ^{177}Lu -TEFAPI-06 and ^{177}Lu -TEFAPI-07 in saline and human serum were monitored from 2 h to 168 h using radio-HPLC. The chromatograms were collected on a XBridge C18 column (4.6 $\times$ 150 mm, 5 μm , Waters) at 35 °C. Water with 0.1% TFA was used as mobile phase A and acetonitrile with 0.1% TFA was used as mobile phase B, the gradient condition was: 0-2 min, 10% B; 2-10 min, 10%-60% B; 10-12 min, 60% B; 12-15 min, 60%-10% B, and the flow rate was 1 mL/min.

Supplemental Fig 22. In vitro stability analysis of ^{177}Lu -TEFAPI-06 and ^{177}Lu -TEFAPI-07.

4. Results of cell assays

Supplemental Fig 23. (A) Competition assay of FAPI-04; (B) Saturation binding assay of ⁶⁸Ga-FAPI-04. (C) Competition assays of TEFAPI-06 and TEFAPI-07 in the absence of albumin; (D) Saturation binding assays of radiolabeled TEFAPI-06 and TEFAPI-07 in the absence of albumin; (E) Cellular uptake assays of ⁶⁸Ga-TEFAPI-06 and ⁶⁸Ga-TEFAPI-07 in HT-1080-FAP and HT-1080 cells in the presence of albumin.

5. PET imaging results of ^{86}Y -FAPI-04 in PDX-bearing mice

Supplemental Fig 24. (A-C) PET imaging of ^{86}Y -FAPI-04 (A), ^{86}Y -TEFAPI-06 (B) and ^{86}Y -TEFAPI-07 (C) at the indicated time points post injection in PDX-bearing mice. (D) The time-activity curves for tumor of ^{86}Y -FAPI-04, ^{86}Y -TEFAPI-06 and ^{86}Y -TEFAPI-07.

(E) The area under the curve (AUC) of ^{86}Y -TEFAPI-06 and ^{86}Y -TEFAPI-07 in tumor is significantly higher than the AUC of ^{86}Y -FAPI-04, respectively.

6. PET imaging results of HT-1080-FAP and HT-1080 tumor bearing mice

Supplemental Fig 25. (A) PET imaging of ^{86}Y -TEFAPI-06 in HT-1080-FAP and HT-1080 tumor bearing mice; (B) Time-activity curve and area under the curve for HT-1080-FAP and HT-1080 tumor

Supplemental Fig 26. (A) PET imaging of ^{86}Y -TEFAPI-07 in HT-1080-FAP and HT-1080 tumor bearing mice; (B) Time-activity curve and area under the curve for HT-1080-FAP and HT-1080 tumor

7 Blocking study results of TEFAPI-06 or TEFAPI-07

Supplemental Fig 27. (A) Blocking study by pre-treated with non-radiolabeled TEFAPI-06 or TEFAPI-07. (B) PET imaging of ^{68}Ga -FAPI-04 in PDX-bearing mice at 30 min post injection with or without the treatment of TEFAPI-06 or TEFAPI-07. (C) A series of PET images have been recorded before or at 12 h, 24 h after treatment with TEFAPI; The corresponding ROI analysis of tumor uptake with or without the treatment of TEFAPI-06 or TEFAPI-07.

8. SPECT imaging results of ^{177}Lu -TEFAPI-06 and ^{177}Lu -TEFAPI-07

Supplemental Fig 28. SPECT imaging results of ^{177}Lu -TEFAPI-06 and ^{177}Lu -TEFAPI-07 using pancreatic PDX tumor bearing mice.

9. Results of histopathologic staining

Supplemental Fig 29. H&E staining of excised main organs including heart, liver, spleen, lung and kidney after treatment with ^{177}Lu -TEFAPI-06, ^{177}Lu -TEFAPI-07 and saline, respectively.