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Bioluminescent Probe for Monitoring Endogenous Fibroblast Activation Protein-Alpha

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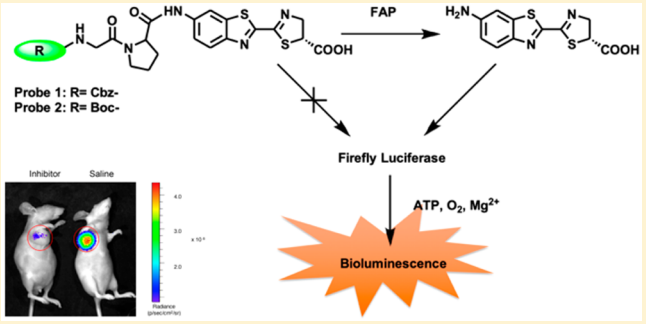
Supporting Information

ABSTRACT: Fibroblast activation protein-α (FAP), as a crucial member of cell surface glycoprotein, highly expresses in reactive fibroblasts of tumors and several fibrosis diseases. It is a potential target for drug design and also reported as a prodrug strategy to increase the therapeutic window of some anticancer agents. In this work, we developed the first bioluminogenic probe for FAP with a limit-of-detection of 0.254 ng/mL, which could be applied to evaluate the FAP inhibitors in vitro. The experiments of transgenic mice and tumor-bearing nude mice validated our probe 1 could reflect the endogenous FAP level in vivo. Furthermore, this probe was successfully used to reflect FAP up-regulation in the lung homogenates of the bleomycin-induced idiopathic pulmonary fibrosis mice.



Fibroblast activation protein-α (FAP, FAPα), a member of dipeptidyl peptidase (DPP) family, pocesses the enzymatic activity of both dipeptidyl peptidase and endopeptidase.[1] FAP has been reported to overexpress in more than 90% of epithelial cancers and affect the proliferation as well as invasion of tumors.[2,3] Hence, it is accepted as a biomarker of tumor stromal fibroblasts and potential drug target. What's more, some diseases linked with activated stroma, such as fibrosis diseases and wound healing, also promote the expression of FAP.[4,5] Therefore, it is related to tissue remodeling as well. Considering the essential biological functions of FAP, it is of significance to establish a new analytical method with high sensitivity and selectivity for FAP detection.

So far, there were several fluorescent probes utilized for FAP detection.[6−11] For example, Ma reported a fluorescent probe for FAP and applied it for confocal imaging of FAP in living cells.[6] Pu developed one for successfully imaging the overexpression of FAP in wound borders of keloids,[10] while Wu described one for tumor imaging in nude mice model.[11]

Compared with fluorescent probes, probes utilized the bioluminescence do not need excitation lights, thus displaying better signal-to-noise ratio. Caging the key reactive site between luciferin and firefly luciferase (Fluc) could effectively quench the bioluminescence. Many probes were developed based on this strategy.[12−23] In the first step, target biomolecules cleave the caged group and release luminophore, which is the same as the fluorescent one. Subsequently, firefly luciferase catalyzes the oxidation of luciferin and emits bioluminescence immediately. In this way, the signal can be amplified and detected easily.

EXPERIMENTAL SECTION

Synthesis. The synthetic routine of probes 1 and 2 was depicted in Scheme S1. The detail of synthesis was described in the Supporting Information.

Reagents. All reagents and solvents available from commercial sources were used without further purified unless otherwise noted. *N*-Carbobenzyloxy-Gly-Pro-OH and *N*-*tert*-butoxycarbonyl-Gly-Pro-OH were purchased from Changzhou Kanglong Biotech Ltd. *N*-Methylmorpholine, *i*-BuOCOCl were purchased from Energy Chemical Co., China. γ-Glutamyl transpeptidase (GGT), aminopeptidase N (APN), fibroblast activation protein (FAP), dipeptide peptidase IV (DPPIV), and tyrosinase (TYR) were purchased from Sigma-Aldrich. SP-13786 was purchased from Medchemexpress Co., China. Firefly luciferase was purchased from Promega company. Doubly distilled water was purified with a Milli-Q filtration system for biological experiments in this work.

Bioluminescence Assay Instrumentation. The assays referred to the previously published article.[23] The buffer solution used for the bioluminescence assay was Tris-HCl

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buffer (10 mM, pH 7.4) containing 10 mM $MgCl_2$. Bioluminescent imaging was performed by an IVIS Kinetic imaging system (Caliper Life Sciences, Hopkinton, Massachusetts, U.S.A.). Ring-shaped regions of interest were made and quantified by the software of an IVIS Kinetic imaging system.

Bioluminescence Assay In Vitro. Varied concentrations of fibroblast activation protein (FAP) solutions (50 μL) wee added to the solutions of probes 1 and 2 (50 μL, 20 μM) in 96-well plates and incubated at 37 °C for 1.5 h. Then 50 μL of Tris-HCl buffer containing 100 μg/mL firefly luciferase, 10 mM $MgCl_2$, and 2 mM ATP was added to each well at the same time. The bioluminescent images were captured immediately by the living imaging system, and the bioluminescent intensity was quantified by its software. The relative total photo flux was obtained from dividing the bioluminescence intensity of different analytes by the blank.

The 1 mM solutions of inorganic salts, different biomolecules such as glucose, reduced glutathione, and D-cysteine, as well as Vanin-1 (30 ng/mL), GGT (50 U/L), aminopeptidase N (50 U/L), DPPIV (125 ng/mL), and FAP (125 ng/mL) were added and incubated at 37 °C for 1.5 h. Then 50 μL of Tris-HCl buffer containing 100 μg/mL firefly luciferase, 10 mM $MgCl_2$, and 2 mM ATP was added to each well at the same time. The bioluminescent images were captured immediately by the living imaging system and the bioluminescent intensity was quantified by its software.

Cytotoxicity Assay. The cytotoxicity of probe 1 and SP-13786 (the inhibitor of FAP) was performed with a standard CCK8 method on U87MG cells expressing firefly luciferase (U87MG-Fluc). U87MG-Fluc cells were plated in 96-well plates with DMEM (10% fetal bovine serum) overnight. Then 100 μL of solution of different concentrations of probe 1 and the inhibitor were added each well and incubated for 3h. CCK8 method was performed after the removal of medium.

Bioluminescence Imaging in Cellulo. A total of 100 μL of U87MG-Fluc cell suspension in DMEM medium with 4×10^5 cells per mL was plated to the 96-well plates (Corning, 3603) and cultured overnight. The medium was removed, then 50 μL of SP-13786 (50 and 100 μM) was added and incubated for 30 min. Then, 40 μM of probe 1 solution was added. The bioluminescent images was aquired and the bioluminescent intensity was quantified by the software of living imaging system.

Bioluminescence Imaging In Vivo. All animal studies were approved by the Ethics Committee and IACUC of Cheeloo College of Medicine, Shandong University, and were conducted in compliance with European guidelines for the care and use of laboratory animals. Pathogen-free luciferase-expressing transgenic mice (FVB-Tg(CAG-Fluc,-GFP)-L2G85Chco/FathJ17) were obtained from the Jackson Laboratory.

FVB-Fluc+ mice were divided into two groups. They were injected with SP-13786 in saline (5 mg/kg) or an equal amount of vehicle intravenously (i.v.), respectively. One hour later, all mice were injected with probe 1 i.v. (1 mM, 100 μL). The bioluminescent signal was recorded by IVIS Kinetic imaging system for 1 to 30 min.

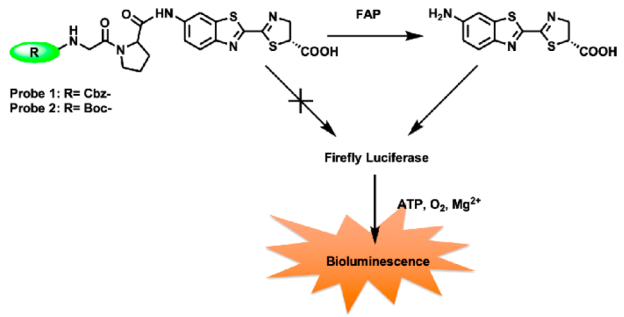
U87MG-Fluc cells were grafted subcutaneously under the right forelimb armpit of BALB/c nude mice. Two weeks later tumor volume reached about 400 mm^3. The mice were divided into two groups. One group was injected with SP-13786 in saline (5 mg/kg) i.v., and the other was injected with an equal amount of vehicle. After 60 min, probe 1 (4 mM, 200 μL) was injected intraperitoneally (i.p.). The bioluminescent signal was recorded by IVIS Kinetic imaging system for 1−100 min.

Applications in Bleomycin-Induced Idiopathic Pulmonary Fibrosis (IPF) Model. Bleomycin (BLM) was utilized to induce IPF of Kunming mice. For one group, BLM was i.p. injected at a concentration of 7.5 mg/kg for 10 d. The other group was injected with an equal amount of saline. At 29 d after the last administration, the mice were sacrificed and lungs were harvested. Hematoxylin and eosin, as well as Masson staining were performed to confirm the form of IPF. Immunohistochemistry staining and Western blot analysis were also performed to prove the high expression of FAP in IPF model. Lungs were homogenized in potassium phosphate buffer with 0.1% Triton X-100 and 0.4% proteasome. Probe 1 (20 μM) was incubated with the protein solution (10 μg/mL total protein) for 90 min in 96-well plates. Then a solution of luciferase was added to each well and bioluminescent image was acquired immediately.

RESULT AND DISCUSSION

In this work, we employed *N*-carbobenzyloxy-Gly-Pro-OH (Cbz-Gly-Pro-OH, probe 1) and *N*-*tert*-butoxycarbonyl-Gly-Pro-OH (Boc-Gly-Pro-OH, probe 2) as the recognition groups of FAP (Scheme 1). Conjugating these units with the aminoluciferin could hinder its recognition with firefly luciferase (Fluc). The stability of probe 1 has been studied by HPLC system in 150 min, which indicated that our probe was stable in the test conditions (Figure S1). Fibroblast activation protein (FAP) could cleave the amide bond after the proline and release aminoluciferin, a substrate of Fluc, which has been validated by the HPLC analysis (Figure S2). What's more, the identity of the product peak was confirmed by ESI-MS (Figure S3). Subsequently, bioluminescence was generated via the reaction with Fluc in the presence of adenosine triphosphate (ATP), Mg^{2+}, and O_2 as depicted in Scheme 1. The synthetic details of probes were presented in Scheme S1, as depicted in the Supporting Information.

Scheme 1. Design Strategy for the Probes of FAP



Initial studies of probes mostly focused on their efficiency in an aqueous environment at the physiological condition in vitro. In brief, different concentration of FAP was incubated with a solution of probes (20 μM) for 90 min at physiological condition (37 °C and pH = 7.4) in a 96-well black microplate. In such a case, recognition groups would be first cleaved to release aminoluciferin. Subsequently, 50 μL of 0.1 mg/mL Fluc solution containing 1 mM ATP was added to each well simultaneously, the bioluminescence was observed by an IVIS Kinetic imaging system. Both probes 1 and 2 displayed apparent bioluminescence augmentation at the FAP concen-

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tration of 500 ng/mL, which was about 185- and 75-fold increasing compared to the blank, respectively (Figure 1B).

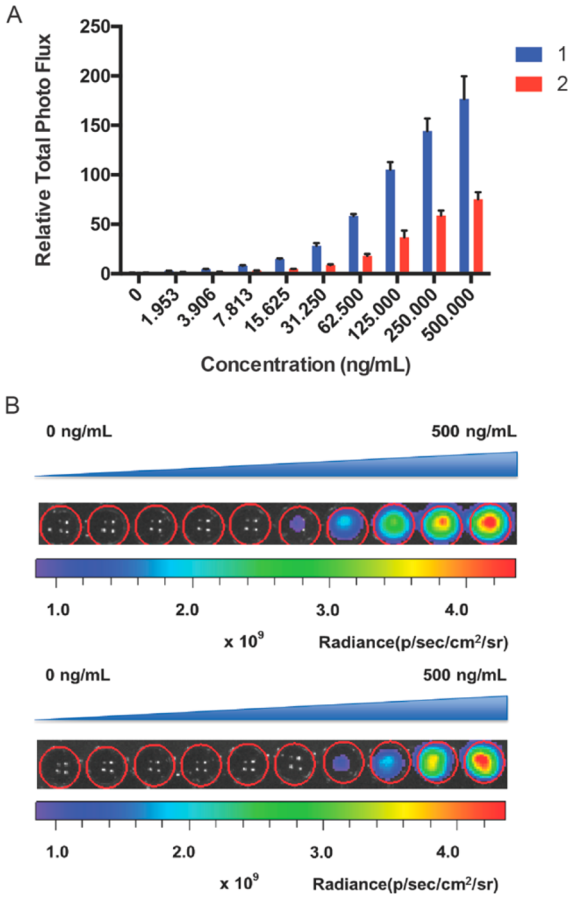

Figure 1. (A) Relative total photo flux change of probes (20 μM) with different concentration of FAP after 90 min incubation; (B) Bioluminescence image of part A.

Moreover, probes 1 and 2 provided ideal linearity at the range from 0 to 125 ng/mL ($Y = 0.845X + 1.735$, $R^2 = 0.993$, Figure S4) and from 0 to 62.5 ng/mL ($Y = 0.270X + 0.801$, $R^2 = 0.984$, Figure S5), respectively. These linear equations could be used to quantify the concentration of FAP in vitro. The limit of detection (LOD) of probes 1 and 2 was 0.254 and 0.419 ng/mL, respectively, which was calculated by the general $3\sigma/k$ method, where σ is the standard deviation of the blank sample and k is the slope of the linear regression equation. We also obtained the Michaelis constant (K_m) and turnover number (k_{cat}) to be 29.7 μM and 0.0548 s^{-1} for probe 1 (Figure S6) and 22.4 μM and 0.0320 s^{-1} for probe 2 (Figure S7), respectively, by using the Michaelis−Menten equation. Overall, these results indicated the good reactivity of the probes.

Subsequently, $FeCl_3$, $MgCl_2$, $BaCl_2$, $MnSO_4$, $CaCl_2$, $ZnCl_2$, $AlCl_3$, $PbCl_2$, $CuSO_4$, and reduced glutathione (GSH), D-cysteine (D-Cys), glucose, homocysteine (Hcy), Vanin-1, γ-glutamyl transpeptidase (GGT), aminopeptidase N (APN), and dipeptide peptidase IV (DPPIV) were selected to evaluate the selectivity of probes toward FAP. As a result, all probes indicated negligible bioluminescent response to the inorganic salts (1 mM, Figure S8). However, probe 2 displayed an apparent response to DPPIV, which was about 22-fold compared with the blank control at DPPIV concentration of 125 ng/mL. The response of probe 2 toward FAP was only about 2-fold higher than its response toward DPPIV at the same concentration as depicted in Figure 2B. Therefore, we thought probe 1 possessed higher sensitivity and selectivity than probe 2, which can be utilized for further bioluminescent imaging in cellullo and in vivo.

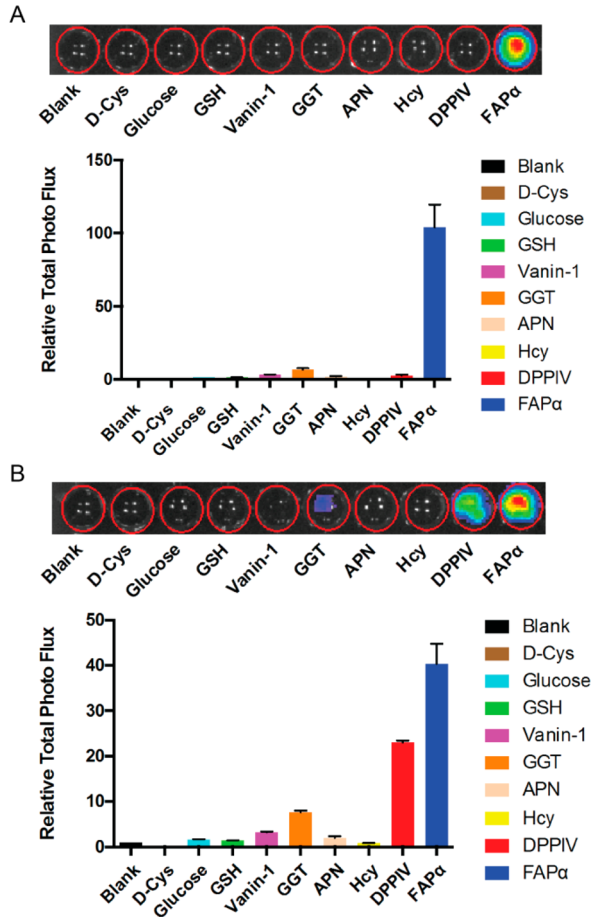

Figure 2. (A) Bioluminescence imaging of probe 1 (20 μM) incubated with different biomolecules (1 mM), Vanin-1 (30 ng/mL), GGT (50 U/L), aminopeptidase N (50 U/L), DPPIV (125 ng/mL), and FAP (125 ng/mL); (B) Bioluminescence imaging of probe 2 (20 μM) incubated with different biomolecules.

Given the preferred performance of probe 1, we try to employ it to evaluate the FAP inhibitor, SP-13786, which was commercially available now.[24] Before this experiment, we examine whether SP-13786 would have an inhibition effect on Fluc. After incubation of firefly luciferase with 100 and 200 μM of SP-13786 for 60 min, a solution of aminoluciferin was added. The bioluminescent intensity was almost the same as the blank group without inhibitor, which indicated that SP-13786 had a negligible influence on the activity of luciferase (Figure S9). To a solution of 50 ng/mL FAP, different concentrations of SP-13786 was added, and the mixture was incubated for 60 min at 37 °C. Thereafter, a solution of probe 1 was added and incubated for 90 min. Then the buffer solution containing firefly luciferase, $MgCl_2$, and ATP was added. The bioluminescent images were captured and the bioluminescent intensity was quantified. (Figure 3A,B). The IC_{50} value by using this method was 3.56 ± 1.05 nM, which is consistent with the literature (Figure S10).[24] In bioluminescence assays in vitro, an excess of luciferase is added after the

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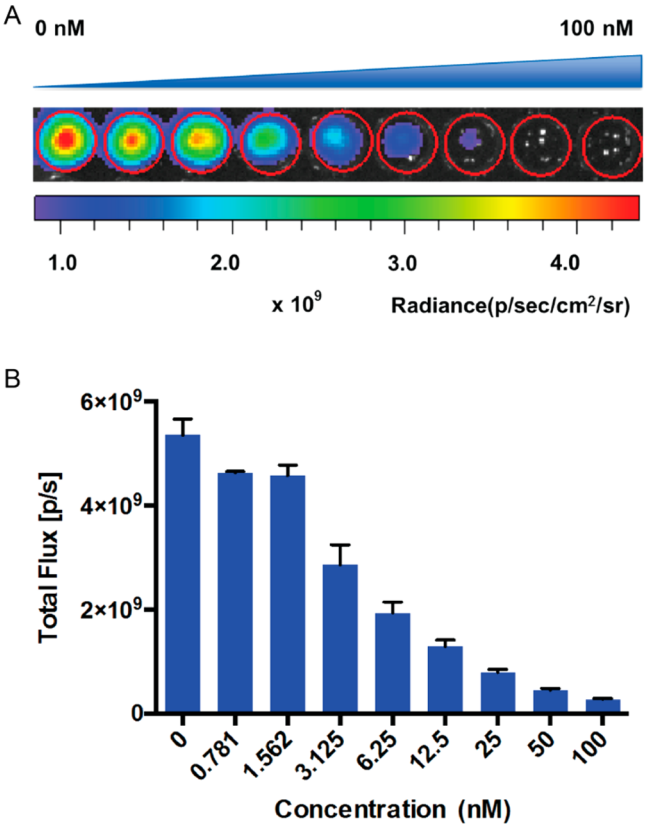

Figure 3. (A) Bioluminescence image of 50 ng/mL FAP incubated with different concentrations of SP-13786 for 60 min, then probe 1 (20 μM) was added to the mixture; (B) Quantification of the part A.

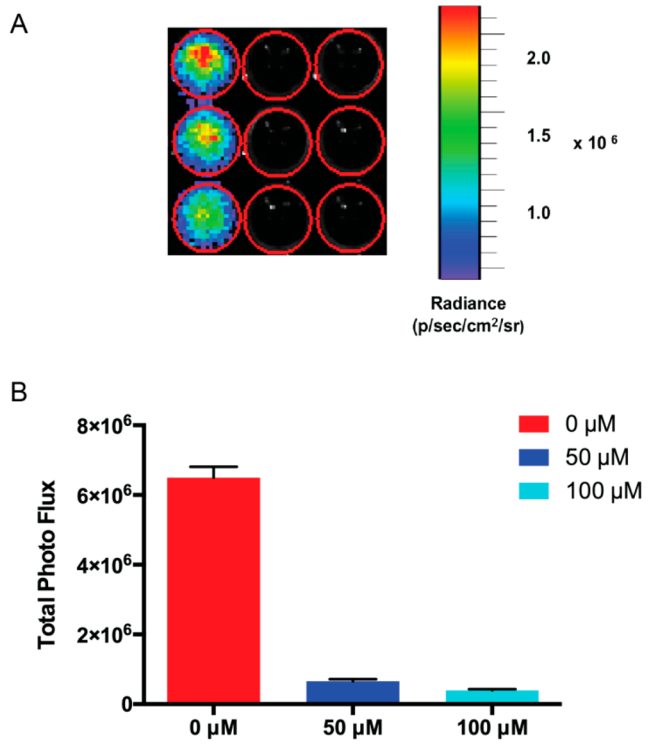

Figure 4. (A) Bioluminescence intensity of U87MG-Fluc cells incubated with SP-13786 (0 μM, 50 μM, 100 μM) for 30 min, then probe 1 was added to each well; (B) Quantification of part A.

FAP-catalyzed reaction of probes; therefore, the accuracy of FAP detection will not be influenced by luciferase-catalyzed reaction.

After successfully confirming the selectivity and sensitivity of probe 1 in vitro, we applied probe 1 for extensive study in living cells. U87MG-Fluc cell line was chosen for the experiment in cellullo because of its high expression of FAP.[9] To examine the performance of probe 1 for detecting endogenous FAP in living-cell assays, SP-13786 was engaged to decrease the intracellular FAP level. Before the experiments in living cells, CCK-8 assay was performed to test the cell viability of probe 1 and inhibitor SP-13786. U87MG cells were incubated with probe 1 and inhibitor for 180 min, and this compound showed little cytotoxicity at the range from 4 to 250 μM (Figures S11 and S12). In addition, SP-13786 had negligible influence on the bioluminescence of aminoluciferin in this cell line (Figure S13). After incubating U87MG cells with the inhibitor for 30 min, 40 μM of probe 1 solution was added. The bioluminescence of probe 1 decreased significantly, which was only about 10% (50 μM SP-13786) and 5% (100 μM SP-13786) of the control group (Figure 4). All these data confirmed the efficiency of probe 1 to reflect the variance of intracellular FAP.

Since our probe has been confirmed have outstanding performance in vitro and in cells, we subsequently utilized it for the bioluminescent imaging of living mice. It has been reported that the intravenous (i.v.) administration of SP-13786 would decrease FAP activity in vivo.[24] We hypothesized that the suppressed FAP activity induced by the inhibitor would decrease the conversion from the probe to aminoluciferin; thus bioluminescent intensity would be weakened. FVB-luc+ mice were divided into two groups. They were injected with SP-13786 in saline (5 mg/kg) or an equal amount of vehicle intravenously (i.v.), respectively. One hour later, all mice were injected with probe 1 i.v. (1 mM, 100 μL). The bioluminescent signal was recorded by an IVIS Kinetic imaging system for 30 min (Figure S14). As displayed in Figure 5C, the bioluminescent intensity of both groups reached its peak at 2 min and decreased slowly after that. The saline group was about 5 times the bioluminescent intensity of the inhibitor group at 20 min after injection of the probe (Figure 5A,B). Additionally, we constructed the tumor-bearing mice model by xenograft U87MG-Fluc cells to nude mice. U87MG-Fluc cells were grafted subcutaneously under the right forelimb armpit of BALB/c nude mice. Two weeks later, the tumor volume reached about 500 mm^3. The mice were divided into two groups. One group was injected with SP-13786 in saline (5 mg/kg) i.v., and the other was injected with an equal amount of vehicle. After 60 min, probe 1 (4 mM, 200 μL) was injected intraperitoneally (i.p.). As depicted in Figure 5F, the bioluminescent intensity reached its peak at about 80 min and apparently decayed after that. Time-dependent bioluminescent images after injection of the probe were recorded (Figure S15). The bioluminescence of mice injected with a vehicle was about 4-fold higher than the group injected with inhibitor after the administration of probe at 65 min. All these data above revealed that our probe could reflect the endogenous FAP level in the living mice model.

It has been reported that FAP is overexpressed in idiopathic pulmonary fibrosis (IPF). Bleomycin (BLM) was utilized to induce IPF of Kunming mice. For one group, BLM was i.p. injected at a concentration of 7.5 mg/kg for 10 d as the previous report.[25] The other group was injected with an equal

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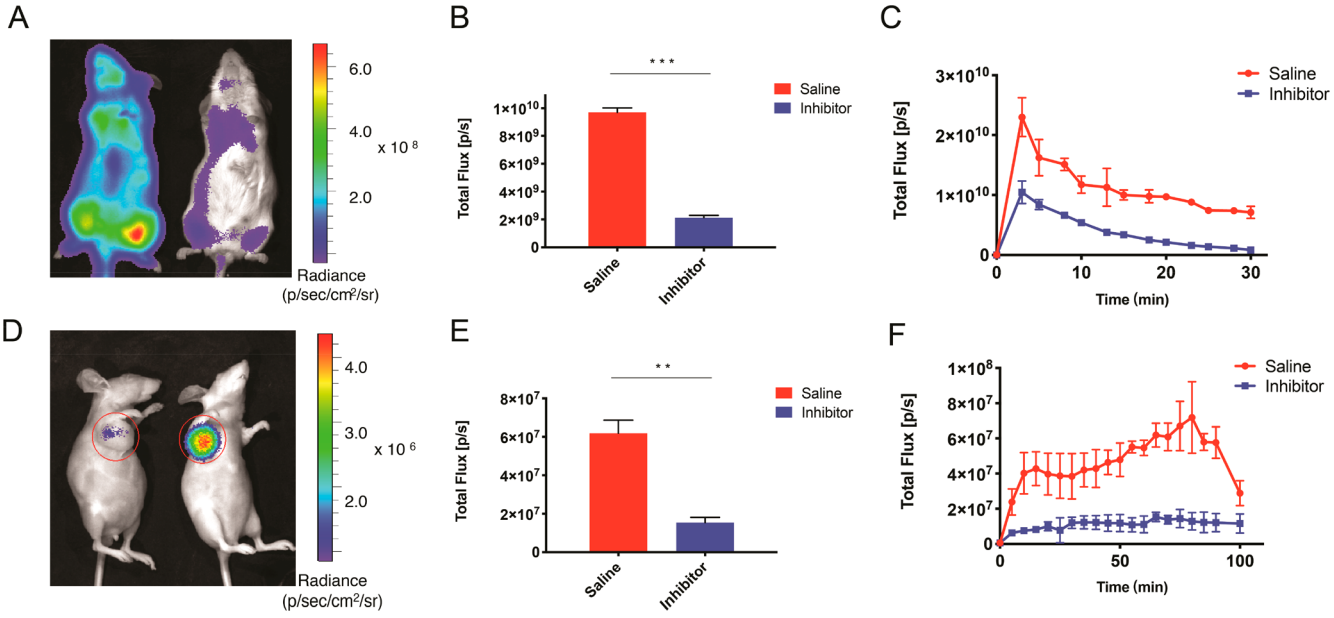

Figure 5. FVB-luc+ mice were divided into two groups. One was injected with SP-13786 in saline (5 mg/kg) i.v. and the other was injected with an equal amount of vehicle. Then, probe 1 (1 mM, 100 μL) was injected i.v. (A) Bioluminescent imaging of transgenic mice after injection of probe 20 min; (B) Quantification of part A ($n \geq 3$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ vs control); (C) The change of bioluminescent intensity after injection of probe; Nude mice bearing U87MG-Fluc cells preinjected with SP-13786 in saline (5 mg/kg) i.v. and an equal amount of saline i.v.; (D) Bioluminescent imaging of nude mice after injection of probe 65 min; (E) Quantification of part D; (F) The change of bioluminescent intensity after injection of the probe.

amount of saline. At 29 d after the last administration, the mice were sacrificed and lungs were harvested. Hematoxylin and eosin, as well as Masson staining, displayed that BLM had induced IPF of mice on that day (Figure S16C–F). FAP was found to be overexpressed in an IPF lung by immunohistochemistry staining (Figure S16A,B) and Western blot analysis (Figure S17). Probe 1 was applied for the detection of FAP in mice lung homogenates. The total protein level of the BLM and saline groups was quantified by bicinchoninic acid (BCA) assay. A total of 20 μM of probe 1 was added to 10 μg/mL total protein. After incubation for 90 min, Fluc was added to each well, and bioluminescence was acquired immediately. As shown in Figure S18, the bioluminescent intensity of BLM-induced group was obviously higher than the saline group.

CONCLUSION

In summary, by introducing FAP recognition moieties to aminoluciferin, we developed two probes for FAP. Considering the LOD of probe 1 was as low as 0.254 ng/mL, this probe was used to evaluate the inhibition ability of the commercial FAP inhibitor. After an extensive in vitro evaluation, probe 1 with higher sensitivity and selectivity was selected for further evaluations in living cells and living mice. These in cellulo and in vivo experiments indicated our probe could reflect the endogenous FAP level. Moreover, this probe was successfully applied for tissue homogenates of IPF mice. By expanding our ability to monitor endogenous FAP level from cell to living animals, our probe is expected to provide a unique chemical tool to sensitively map the FAP level, and to advance our understanding biological contribution of this protein.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.9b02117.

Full experimental procedure, NMR, and MS spectra (PDF)

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The manuscript was written through contributions of all authors.

Notes

The authors declare no competing financial interest.

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