SUPPORTING INFORMATION

Bioluminescent Probe for Monitoring Endogenous Fibroblast Activation Protein Alpha

Yuxing Lin, 1,3 Zhao Ma, 1 Zhenzhen Li, 1 Yuqi Gao, 1 Xiaojun Qin, 1 Zheng Zhang, 1 Guankai Wang, 1 Lupei Du, 1 Minyong Li1,2,*

1Department of Medicinal Chemistry, Key Laboratory of Chemical Biology (MOE), School of Pharmacy, Shandong University, Jinan, Shandong 250012, China. Tel./fax: +86-531-8838-2076; E-mail: mli@sdu.edu.cn
2State Key Laboratory of Microbial Technology, Shandong University, Jinan, Shandong 250100, China
3Institute of Medical Sciences, the Second Hospital of Shandong University, 247# Beiyuan Street, Jinan, Shandong 250033, PR China.

CONTENT

1. General methods..................................................................................................................S-2
2. Synthesis .............................................................................................................................S-3
3. Experiment graphs ..............................................................................................................S-4
4. NMR and MS spectra........................................................................................................S-15
1. General methods

All chemicals and solvents were used without purification unless otherwise noted. Column chromatography was carried out on silica gel (200-300 mesh) using an eluent of ethyl acetate and cyclohexane ether. TLC analyses were conducted on silica gel plates; products were visualized using UV light (ZF-2 UV254) and I₂. Mass spectral analyses were performed on an API 4000 (ESI-HRMS). NMR spectra were recorded at ¹H (400 MHz) and ¹³C (100 MHz) on a Bruker instrument. Chemical shifts (δ values) and coupling constants (J values) are given in ppm and hertz respectively, using solvents (¹H NMR, ¹³C NMR) as the internal standard in DMSO and CDCl₃ solution.

All reagents and solvents available from commercial sources were used without further purified unless otherwise noted. γ-Glutamyl transpeptidase (GGT), aminopeptidase N (APN) and tyrosinase (TYR) were purchased from Sigma-Aldrich. Fibroblast Activation Protein Alpha (FAP), dipeptide peptidase IV (DPPIV), primary antibody of FAP were purchased from Abcum. Water used for all biological studies was doubly distilled and further purified with a Milli-Q filtration system. Buffer solution used in the bioluminescent assay was the Tris-HCl buffer (pH 7.4) comprising 10 mM MgCl₂. The IVIS Kinetic imaging system (Caliper Life Sciences, Hopkinton, Massachusetts, U.S.A.) equipped with a cooled charge-coupled device (CCD) camera was manipulated for the bioluminescent imaging. Firefly luciferase was purchased from Promega company. Circular ROIs were drawn over the areas and quantified using Living Image software (Caliper Life Sciences, Hopkinton, Massachusetts, U.S.A.). U87MG cells expressing firefly luciferase (U87MG-Fluc) were cultivated in DMEM (10% fetal bovine serum at 37 °C. 100 μL of cell suspension with 4×10⁵ cells per mL was added to the 96-well plate (Corning, 3603).
2. Synthesis

Probes 1 and 2 were prepared according to Scheme S1.

![Scheme S1](image)

**Scheme S1.** Synthetic route of probes 1 and 2

**General procedure for N-Acylation of 6-amino-2-cyanobenzothiazole (Method A):** The protected peptides were commercially available. To a solution of 1c or 2c (0.68 mmol) was added N-methylmorpholine (87 μL, 0.68 mmol) and i-BuOCOCl (75 μL, 0.68 mmol) in tetrahydrofuran (2 mL), the mixture was stirred at 0 °C for 0.5 h. Then 2-cyano-6-aminobenzothiazole (40 mg, 0.23 mmol) in 2 mL tetrahydrofuran was added. The mixture was stirred at room temperature overnight. Then the solution was evaporated under reduced pressure. The mixture was diluted with ethyl acetate and (50 mL) washed with saturated sodium bicarbonate solution, water and brine, dried over anhydrous MgSO$_4$ and filtered. The crude product was purified by chromatography with ethyl acetate-hexane (1:2) as the eluents to afford a white solid

**General procedure for cyclization of 2-cyanobenzothiazoles (Method B):** To a solution of 1b or 2b (0.1 mmol) in 2 mL of MeOH was added the mixture of D-cysteine hydrochloride (46 mg, 0.15 mmol) and potassium carbonate (40 mg, 0.3 mmol) dissolved in 2 mL of H$_2$O. The mixture was stirred at room temperature for 10 min. Then, the organic solvents were removed under reduced pressure. 20 mL water was added to the solution and acidified with 1 M HCl solution to afford the product.

**Benzyl(2-(2-((2-cyanobenzo[d]thiazol-6-yl)carbamoyl)pyrrolidin-1-yl)-2-oxoethyl)carbamate (1b)** Compound 1b was synthesized from 1a by Method A to give a white solid (y. 41%) and was applied for the next step without further purification.

**Tert-butyl(2-(2-((2-cyanobenzo[d]thiazol-6-yl)carbamoyl)pyrrolidin-1-yl)-2-oxoethyl)carbamate (2b)** Compound 2b was synthesized from 2a by Method A to give a white solid (y. 36%) and was applied for the next step without further purification.

**(5R)-2-(6-1-(((benzyloxy)carbonyl)glycyl)pyrrolidine-2-carboxamido)benzo[d]thiazol-2-yl)-4,5-dihydrothiazole-5-carboxylic acid (1)** Probe 1 was synthesized from 1b by Method B to give a yellow-white solid (y. 55%). $^1$H-NMR (400 MHz, DMSO) δ 10.42 (s, 1H), 8.60 (s, 1H), 8.10 (d, $J = 9.0$ Hz, 1H), 7.69 – 7.62 (m, 1H), 7.35 (t, $J = 4.2$ Hz, 5H), 5.44 (t, $J = 9.0$ Hz, 1H), 5.03 (s, 2H),...
4.53 – 4.46 (m, 1H), 3.99 – 3.68 (m, 5H), 2.18 – 1.89 (m, 4H). $^{13}$C-NMR (101 MHz, DMSO) δ 171.60, 167.87, 164.88, 159.50, 156.98, 149.04, 138.85, 137.56, 136.74, 128.80, 128.23, 128.13, 124.66, 120.12, 111.96, 78.58, 65.84, 60.90, 46.37, 43.20, 35.24, 29.75, 24.96. ESI-MS: m/z [M+H$^+$] calcd. for 568.1319, found 568.1315.

(5R)-2-(6-(1-((tert-butoxycarbonyl)glycyl)pyrrolidine-2-carboxamido)benzo[d]thiazol-2-yl)-4,5-dihydrothiazole-5-carboxylic acid (Probe 2) Probe 2 was synthesized from 1b by Method B to give a yellow-white solid (y. 51%). $^1$H-NMR (400 MHz, DMSO) δ 10.38 (s, 1H), 8.59 (d, J = 1.8 Hz, 1H), 8.10 (d, J = 9.0 Hz, 1H), 7.65 (dd, J = 8.9, 1.9 Hz, 1H), 6.84 (t, J = 5.7 Hz, 1H), 5.44 (t, J = 9.0 Hz, 1H), 4.49 (dd, J = 8.2, 3.1 Hz, 1H), 3.76 (ddd, J = 19.8, 12.6, 3.5 Hz, 4H), 3.56 (ddd, J = 23.1, 11.6, 5.7 Hz, 2H), 2.16 (ddd, J = 14.7, 9.2, 5.5 Hz, 1H), 2.05 – 1.90 (m, 4H), 1.37 (s, 9H). $^{13}$C-NMR (101 MHz, DMSO) δ 171.54 (s), 171.31(s) 168.14 (s), 164.88 (s), 159.49 (s), 156.28 (s), 149.05 (s), 138.84 (s), 136.73 (s), 124.65 (s), 120.14 (s), 111.99 (s), 78.77 (s), 78.48 (s), 60.88 (s), 46.36 (s), 42.88 (s), 35.24 (s), 29.70 (s), 28.67 (s), 24.96 (s). ESI-MS: m/z [M+H$^+$] calcd. for 534.1476, found 534.1384.
3. Experiment graphs

Figure S1. The stability of probe 1 has been studied by HPLC system with Tris-HCl buffer (10 mM, pH 7.4) in 150 min. Wavelength for detection: 330 nm.
Figure S2. HPLC analysis of the reaction between compound 1 and FAP: (A) probe 1 (0.2 mM); (B) aminoluciferin (0.2 mM); (C) probe 1 (0.2 mM, 1 mL) with the addition of FAP (200 ng/mL, 1 mL) and incubated for 20 min. Wavelength for detection: 330 nm.

Figure S3. The identity of the product peak was confirmed by ESI-MS. [M+H+] calcd. for 280.0209, found 280.0204.
Figure S4. Linear fitting curve between the relative bioluminescence intensity of the probe 1 and the concentration of FAP, $Y = 0.8445X + 1.735$, ($R^2 = 0.9929$). The detection limit of probe 1 was 0.254 ng/mL ($3\sigma/k$). The LOD was calculated according to the general $3\sigma$ method ($3\sigma/k$). Where $\sigma$ is the standard deviation of the blank sample and $k$ is the slope of the linear regression equation.

Figure S5. Linear fitting curve between the relative bioluminescence intensity of the probe 2 and the concentration of FAP $Y = 0.2703X + 0.8009$, ($R^2 = 0.9844$). The detection limit of probe 2 was 0.418 ng/mL ($3\sigma/k$).
Figure S6. Lineweaver-Burk plot for the enzyme-catalyzed reaction of probe 1. The Michaelis-Menten equation is described as: \( V = \frac{V_{\text{max}} [\text{probe}]}{K_m + [\text{probe}]} \), where \( V \) is the reaction rate, [probe] is the probe concentration (substrate), and \( K_m \) is the Michaelis constant. Points were fitted in a linear regression model (correlation coefficient \( R=0.987 \)).

Figure S7. Lineweaver-Burk plot for the enzyme-catalyzed reaction of probe 2. Points were fitted in a linear regression model (correlation coefficient \( R=0.986 \)).
Figure S8. (A) Bioluminescence imaging of probe 1 incubated with different inorganic salts (1 mM); (B) Bioluminescence imaging of probe 2 incubated with different inorganic salts (1 mM).

Figure S9. The bioluminescent intensity of incubating 100 ng/mL firefly luciferase with 100 μM and 200 μM inhibitor for 60 min, then a solution of aminoluciferin was added.

IC$_{50}$ = 3.56 ± 1.05 nM

Figure S10. IC$_{50}$ values of SP-13786 as mean ± SD (n = 3).
Figure S11. The cell viability of probe 1 at varied concentration (0-250 μM) incubated for 3 h via CCK8 assay.

Figure S12. The cell viability of SP-13786 at varied concentration (0-250 μM) incubated for 3 h via CCK8 assay.
**Figure S13.** Aminoluciferin 10 μM was employed to test if SP-13786 may inhibit the luciferase activity in the bioluminescence cell imaging assay. Bioluminescence intensity of U87MG-Fluc cells incubated with SP-13786 (0 μM, 50 μM, 100 μM).

**Figure S14.** FVB-luc+ mice were divided into two groups. They were injected with SP-13786 in saline (5 mg/kg) or equal amount of vehicle intravenously (i.v.), respectively. One hour later, all mice were injected with probe 1 i.v. (1 mM, 100 μL). Bioluminescent imaging of transgenic mice after the injection of probe.
Figure S15. Tumor-bearing mice were divided into two groups. One group was injected with SP-13786 in saline (5 mg/kg) intravenously (i.v.) and the other was injected with the equal amount of vehicle. After 60 min, probe 1 (4 mM, 200 μL) was injected intraperitoneally. Bioluminescent imaging of nude mice after the injection of probe.
Figure S16. Bleomycin or saline was intraperitoneally given to Kunming mice, and lungs were harvested at 29 days after the final administration of drug. Immunohistochemistry for FAPa in the lung of saline group (A) and BLM group (B). H&E staining of saline (C) and bleomycin (D) lung sections. Trichrome staining of saline (E) and bleomycin (F) lung sections.
Figure S17. Bleomycin or saline was intraperitoneally given to Kunming mice, and lungs were harvested at 29 days after the final administration of drug. Western blot analysis of FAP expression in the lung homogenates of Kunming mice.

Figure S18. (A) Bioluminescent imaging of probe 1 incubated with mice lung homogenates of saline group (left) and bleomycin group (right); (B) Quantification of part A
3. NMR and MS spectra of the final probe

Probe 1
Probe 2