Electronic Supporting Information

An AND-gate bioluminescent probe for precise tumor imaging

Chenchen Wang[a], Yajian Hong[a], Ling Dong[b], Hu Cheng[a], Duo Jin[a], Ronghua Zhao[c], Zian Yu[a], and Yue Yuan[a,*]

[a]Department of Chemistry, University of Science and Technology of China, 96 Jinzhai Road, Hefei, Anhui 230026, China

[b]Department of Chemistry and Chemical Engineering, Hefei Normal University, Hefei, Anhui 230061, China

[c]Center for Biomedical Imaging, University of Science and Technology of China, Hefei, Anhui 230026, China

Correspondence and requests for materials should be addressed to email:

yueyuan@ustc.edu.cn (Y. Yuan)

Contents:

1. General Methods

Experimental Materials.

All the starting materials were obtained from J&K (Beijing, China), Macklin (Shanghai, China), or Aladdin (Shanghai, China). Commercially available reagents were used without further purification, unless noted otherwise. All chemicals were reagent grade or better. Firefly luciferase plasmid was purchased from Yunzhou Biotechnology (Guangzhou, China). Cathepsin L (CTSL) was purchased from Athens Research Technology (USA). Cathepsin B (CTSB) was purchased from Sigma–Aldrich (USA). 2-Cyano-6-hydroxybenzothiazole (CBT) was obtained from Shanghai Chemical Pharm-Intermediate Tech. (China).

Experimental Instruments.

^{1}H NMR and ^{13}C NMR spectra were obtained on a 400 MHz Bruker AV 400. Mass spectra were performed on a single quadrupole mass spectrometer (Jiangsu Skyray Instrument, China) that was equipped with a standard ESI source. Luminescence emission spectra were obtained on a Hitachi F-4600 fluorescence spectrophotometer with the Xe lamp shut off. HPLC analyses were performed on an Agilent 1200 HPLC system equipped with a G1322A pump and an in-line diode array UV detector using an Agilent Zorbax 300SB-C18 RP column with CH_3CN (0.1% of trifluoroacetic acid (TFA)) and water (0.1% of TFA) as the eluent. 4T1 cells were routinely cultured in Roswell Park Memorial Institute 1640 (RPMI 1640, Viva Cell) supplemented with 10% fetal bovine serum at 37 °C, 5% CO_2, and a humid atmosphere. Seven-week-old (weighing 19-20 g) Balb/c mice were used for animal experiments.

Expression of Firefly Luciferase.

The expression and purification protocol of firefly luciferase was performed according to the literature method.[1] Specifically, BL 21 cells were transformed with the appropriate plasmids (pET-6xHis/Luciferase) and cultured in LB medium supplemented with 100 μg/mL ampicillin at 37 °C until their OD600 reached 0.6-0.8, and then induced with 0.5 mM isopropyl-β-d-thiogalactopyranoside (IPTG) at 16 °C for 20 h. After the bacterial cells were precipitated at 5,000 g for 20 min, the pellets were suspended in

lysis buffer (50 mM Tris, 150 mM NaCl, pH 8.0) and sonicated to disrupt the bacterial cells on ice. The His-tagged enzymes were purified using Ni-NTA-agarose (Sangon) equilibrated in TBS buffer (50 mM Tris, 150 mM NaCl, pH 8.0) supplemented with 10 mM imidazole. After the enzyme supernatant was loaded in the Ni-NTA column and rotated for 1 h, the column was washed with 10 times the column volume (CV) of TBS buffer (50 mM Tris, 150 mM NaCl, 10 mM imidazole, pH 8.0) and 5 times the CV of TBS buffer (50 mM Tris, 150 mM NaCl, pH 8.0, 50 mM imidazole). Then the enzyme was eluted out by adding 0.5 time the CV of TBS buffer (50 mM Tris, 150 mM NaCl, 250 mM imidazole, pH 8.0) to the column for six times. Each fraction of the column chromatograph was analyzed with SDS–PAGE. The protein collected from elution after dialysis was used without further purification and the enzyme concentration was determined with the bicinchoninic acid method.

Immunofluorescence Assays.

4T1-fLuc breast cancer cells were seeded in 14 mm round coverslips that were placed in a 24-well plate for 12 h of incubation. After the cell culture medium was removed, the cells were washed three times with 1 mL phosphate buffered saline (PBS) per well. Then the cells were fixed with 4% paraformaldehyde (500 µL per well) for 20 min before washing with PBS for three times, permeabilized with 0.5% Triton X-100 in PBS for 20 min at room temperature, and then washed three times with PBS. The proteins were blocked with 10% goat serum in PBS for 1 h at room temperature, followed by incubation with primary antibodies (CTSL antibody (E-5), sc-390385, CTSB antibody (H-5), sc-365558, obtained from Santa Cruz Biotechnology, Inc., dilution 1:100) in PBS containing 1.5% goat serum overnight at 4 °C. Then the cells were washed three times with PBST (0.05% Tween-20 in PBS) and incubated with secondary antibody (goat anti-mouse IgG-Alexa Fluor 488, obtained from Absin, dilution 1:100) in PBS containing 1.5% goat serum for 2 h at room temperature. After adding 10 µL of antifade mounting medium with DAPI onto clean glass slides, the coverslips were inverted onto the glass slides with nail polish fixation after quintuple washing in PBST to remove secondary antibodies. Finally, the immunofluorescence imaging was performed on a EVOS

M5000 imaging system (Invitrogen™, Thermo Fisher Scientific). All images and channels were placed at the same settings.

MTT Assay.

The cytotoxicity was measured using a 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay with 4T1 cells (non-luciferase transfected). Cells growing in log phase were seeded into 96-well cell culture plates at 3×10^3 per well. The cells were incubated overnight at 37 °C under 5% CO_2. Solutions of **FK-Luc-BH** or **Ac-Luc-EA** (100 μL/well) at concentrations of 12.5, 25, and 50 μM in 100 μL of medium were added to the wells, and incubated for 12, 24, or 48 h at 37 °C under 5% CO_2, respectively. Ten microliters of a solution of 5 mg/mL MTT dissolved in PBS buffer (pH 7.4) was added to each well of the 96-well plate, then 100 μL DMSO was added to each well to dissolve the formazan for 4 h. After detecting the absorption at 490 nm with an enzyme-linked immunosorbent assay (ELISA) reader (Thermo Scientific Varioskan Flash), the following formula was used to calculate the viability of cell growth: viability (%) = (mean of absorbance value of treatment group/mean of absorbance value of control) × 100.

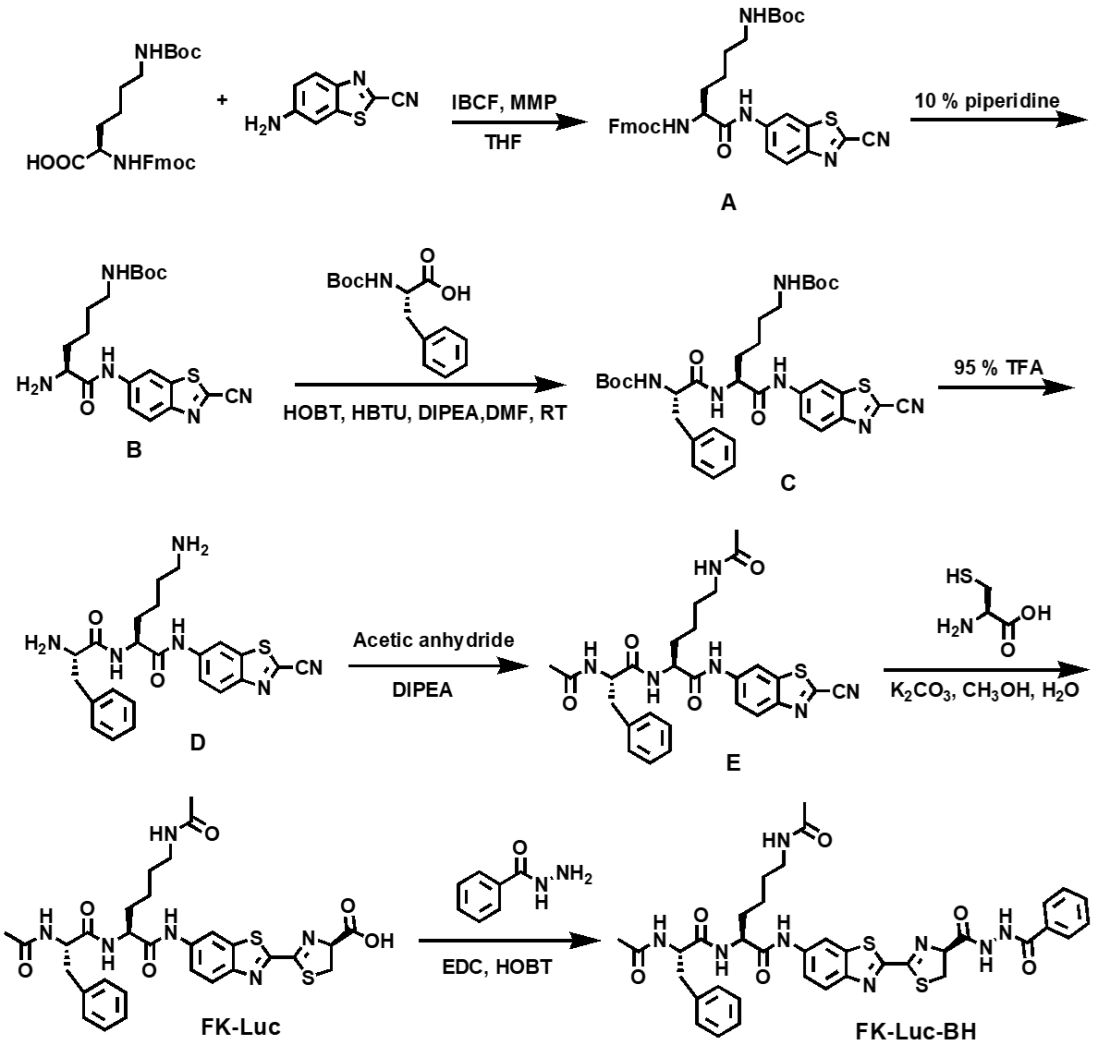
Bioluminescence Imaging of Living Cells and Mice.

Bioluminescence images of cells were acquired using an optical imaging system from Clinx (ChemiScope 6100, Clinx, Shanghai, China). Circular regions of interest (ROIs) were drawn over each well and quantified using ImageJ software. The results were reported as total photon flux within an ROI. For animal imaging, all animals received care according to the guidelines outlined in the Guide for the Care and Use of Laboratory Animals. The procedures were approved by the University of Science and Technology of China Animal Care and Use Committee (Permit Number: USTCACUC23010122026). 3×10^5 4T1-fLuc cells were implanted into the mammary fat pads of 7-week-old Balb/c mice. After 1 week, the tumor-bearing Balb/c mice were injected intraperitoneally with 12.5 μmol/kg **FK-Luc-BH**, 12.5 μmol/kg **Ac-Luc-EA**, and 12.5 μmol/kg **FK-Luc-BH** after the intraperitoneal pre-injection of 3 mmol/kg taurine for 5 min, 0.05 mmol/kg E-64d for 1 h, or both, respectively. Both **FK-Luc-BH** and **Ac-**

Luc-EA were dissolved in PBS/EtOH (11:1) with 0.008% (w/v) Pluronic F-68, taurine or E-64d was dissolved in PBS. *In vivo* BLI was performed at different time points using an *in vivo* optical imaging system (ChemiScope 6100, Clinx, Shanghai, China) equipped with a cooled charge-coupled device (CCD) camera. All mice were anesthetized using isoflurane gas (1.5% isoflurane in oxygen, 1 L/min) during imaging procedures. The pseudocolored bioluminescence images were superimposed over the grayscale photographs of the animals. Circular ROIs were drawn over the tumor areas and quantified using ImageJ software, and the results were reported as total photon flux within an ROI.

2. Syntheses and Characterizations of FK-Luc-BH and Ac-Luc-EA

Scheme S1. Synthetic route for **FK-Luc-BH**.



Synthesis of A: Isobutyl chloroformate (IBCF, 155.6 µL, 1.2 mmol) was added to a mixture of compound Boc-Lys(Fmoc)-OH (570.12 mg, 1.2 mmol) and 4-methylmorpholine (MMP, 219.8 µL, 2 mmol) in THF (4 mL) at 0 °C. The reaction mixture was stirred for 1 h at 0 °C. Then the solution of 2-cyano-6-aminobenzothiazole (CBT, 175.0 mg, 1.0 mmol) was added to the reaction mixture and further stirred for 2 h at 0 °C. After the mixture was stirred overnight at room temperature, compound **A** (438.5 mg, yield: 70%) was purified by HPLC. MS: calculated for **A** $[M + H]^+$: 626.2; obsvd. ESI-MS: m/z 626.9 (Fig. S1).

Synthesis of B: The Fmoc protecting group of compound **A** was cleaved with 10% piperidine in DMF (4 mL) for 5 min at 0 °C, and then 360 µL of TFA was added to

neutralize the alkaline. Compound **B** (263.1 mg, yield: 93%) was obtained by HPLC purification. MS: calculated for **B** $[M + H]^+$: 404.1; obsvd. ESI-MS: m/z 404.3 (Fig. S2).

Synthesis of C: A mixture of compound **B** (202 mg, 0.50 mmol), Boc-Phe-OH (159.18 mg, 0.60 mmol), HBTU (225 mg, 0.60 mmol), and HOBT (81 mg, 0.60 mmol) in DMF (3 mL) was stirred overnight in the presence of DIPEA (104.5 µL, 0.60 mmol). Compound **C** (260 mg, yield: 80%) was obtained after HPLC purification. MS: calculated for **C** $[M + H]^+$: 651.2; obsvd. ESI-MS: m/z 651.3 (Fig. S3).

Synthesis of D: The Boc protecting group of compound **C** was removed with dichloromethane (DCM, 0.5 mL) and triisopropylsilane (TIPS, 50 µL) in TFA (9.5 mL) for 3 h, then the obtained compound **D** (165.6 mg, yield: 92%) was purified by HPLC. MS: calculated for **D** $[M + H]^+$: 451.1; obsvd. ESI-MS: m/z 451.4 (Fig. S4).

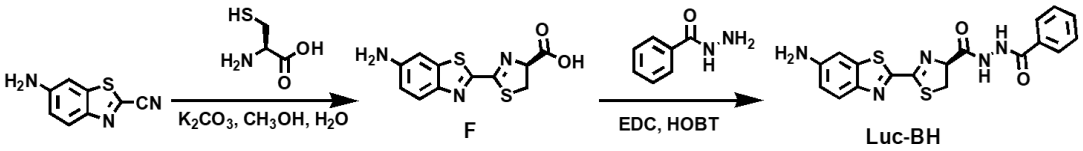
Synthesis of E: A mixture of compound **D** (140 mg, 0.31 mmol) and acetic anhydride (87.9 µL, 0.93 mmol) in DMF (2 mL) was stirred overnight in the presence of DIPEA (80.99 µL, 0.46 mmol). Compound **E** (132 mg, yield: 80%) was obtained after HPLC purification. MS: calculated for **E** $[M + H]^+$: 535.2; obsvd. ESI-MS: m/z 535.3 (Fig. S5).

***Synthesis of FK-Luc*:** $_D$-cysteine hydrochloride monohydrate ($_D$-Cys, 31.2 mg, 0.20 mmol) and compound **E** (100 mg, 0.19 mmol) were suspended in CH_3OH : H_2O (4: 1, v/v, 10 mL) in a 20 mL vial. A saturated solution of potassium carbonate was added to adjust the mixture up to neutral, and the resulting clear solution was stirred for 3 h. Compound **FK-Luc** (103 mg, yield: 86%) was obtained after HPLC purification. MS: calculated for **FK-Luc** $[M + H]^+$: 639.1; obsvd. ESI-MS: m/z 639.0 (Fig. S6).

***Synthesis of* FK-Luc-BH:** Benzohydrazide (16.3 mg, 0.12 mmol)**,** compound **FK-Luc** (65 mg, 0.10 mmol), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI, 28.6 mg, 0.15 mmol), and HOBT (5.4 mg, 0.04 mmol) were suspended in DMF (3 mL) and stirred overnight. Compound **FK-Luc-BH** (30 mg, yield: 40%) was obtained after HPLC purification. ^{1}H NMR (400 MHz, DMSO-d_6): 10.55 (s, 1 H), 10.39 (d, J = 8.2 Hz, 2 H), 8.66 (s, 1 H), 8.29 (d, J = 7.5 Hz, 1 H), 8.12 (dd, J = 8.4, 4.5 Hz, 2 H), 7.90 (d, J = 7.4 Hz, 2 H), 7.82 (t, J = 5.2 Hz, 1 H), 7.67 – 7.55 (m, 2 H), 7.51 (t, J = 7.5 Hz, 2 H), 7.25 (dt, J = 14.9, 7.3 Hz, 4 H), 7.15 (t, J = 6.9 Hz, 1 H), 5.47 (t, J = 9.3 Hz, 1 H), 4.61 – 4.53 (m, 1

H), 4.45 – 4.36 (m, 1 H), 3.85 (t, *J* = 10.5 Hz, 1 H), 3.76 – 3.68 (m, 1 H), 3.35 (s, 5 H), 1.77 (s, 6 H), 1.51 – 1.15 (m, 6 H) (Fig. S12). ^{13}C NMR (100 MHz, DMSO-d_6): 172.13 (C1), 171.56 (C32), 169.84 (C12), 169.45 (C4), 169.01 (C14), 165.82 (C16), 165.35 (C22), 159.56 (C5), 149.11 (C6), 138.69 (C9), 138.46 (C25), 136.91 (C11), 132.79 (C33), 132.39 (C35), 129.60 (C34, C36), 128.99 (C27, C29), 128.47 (C33, C37), 127.90 (C26, C30), 126.67 (C28), 124.75 (C7), 120.22 (C8), 112.00 (C10), 78.32 (C2), 54.35 (C15), 54.13 (C13), 38.77 (C21), 37.95 (C24), 35.09 (C3), 32.03 (C18), 29.32 (C20), 23.39 (C19), 23.08 (C17), 22.96 (C23) (Fig. S13). MS: calculated for **FK-Luc-BH** $[M + Na]^+$: 779.2410; obsvd. ESI-MS: m/z 779.2413 (Fig. S14).

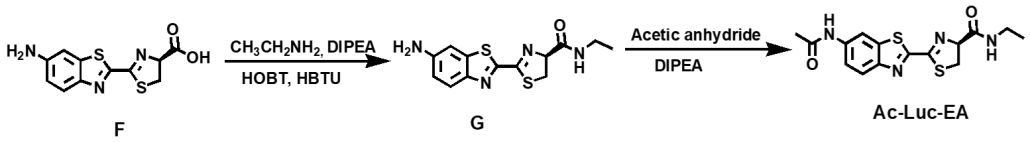
Scheme S2. Synthetic route for **Luc-BH**.



Synthesis of F: $_D$-Cys (60.0 mg, 0.34 mmol) and CBT (56 mg, 0.32 mmol) were suspended in CH_3OH:H_2O (2:1, v/v, 10 mL) in a 20 mL vial. Potassium carbonate (45.4 mg, 0.32 mmol) was then added to the mixture, and the resulting bright yellow-green solution was stirred under N_2 for 20 min. After proving the depletion of CBT by thin layer chromatograph analysis, the methanol was removed in vacuo and the remaining aqueous solution was acidified to pH 3 with 1 M HCl to produce precipitation. Pure $_D$-luciferin (85 mg, yield: 95%) was obtained after filtration and washing with water. MS: calculated for **F** $[M + H]^+$: 280.0, obsvd. ESI-MS: m/z 279.8 (Fig. S9).

Synthesis of Luc-BH: A mixture of compound **F** (15 mg, 0.054 mmol) and benzoylhydrazine (8.8 mg, 0.065 mmol) in DMF (2 mL) was stirred overnight in the presence of HOBT (10.9 mg, 0.081 mmol) and EDCI (15.5 mg, 0.081 mmol). Compound **Luc-BH** (8.6 mg, yield: 40%) was obtained after HPLC purification. MS: calculated for **Luc-BH** $[M + H]^+$: 398.0; obsvd. ESI-MS: m/z 398.5 (Fig. S10).

Scheme S3. Synthetic route for **Ac-Luc-EA**.



Synthesis of G*:* A mixture of compound **F** (50 mg, 0.18 mmol), ethylamine (22 µL, 0.39 mmol), HBTU (146.2 mg, 0.39 mmol), and HOBT (52.6 mg, 0.39 mmol) in DMF (3 mL) was stirred overnight in the presence of DIPEA (67.9 µL, 0.39 mmol). Compound **G** (27.5 mg, yield: 50%) was obtained after HPLC purification. MS: calculated for **G** $[M + H]^+$: 307.0; obsvd. ESI-MS: m/z 306.9 (Fig. S23).

Synthesis of Ac-Luc-EA*:* A mixture of compound **G** (27.5 mg, 0.09 mmol) and acetic anhydride (12.7 µL, 0.135 mmol) in DMF (2 mL) was stirred overnight in the presence of DIPEA (15.6 µL, 0.09 mmol). Compound **Ac-Luc-EA** (13 mg, yield: 41%) was obtained after HPLC purification. ^{1}H NMR (400 MHz, MeOD): 8.63 (m, 1 H), 8.09 (m, 1 H), 7.63 (m, 1 H), 3.35 (m, 3 H), 2.35 (s, 1 H), 2.20 (d, *J* = 3.1 Hz, 3 H), 1.18 (m, 3 H) (Fig. S24). ^{13}C NMR (100 MHz, MeOD): 170.95 (C3), 170.48 (C14), 166.40 (C6), 166.06 (C7), 149.25 (C8), 138.18 (C11), 136.82 (C13), 123.85 (C9), 119.58 (C10), 111.60 (C12), 79.35 (C4), 34.71 (C2), 34.15 (C5), 22.59 (C15), 13.48 (C1) (Fig. S25). MS: calculated for **Ac-Luc-EA** $[M + H]^+$: 349.0; obsvd. ESI-MS: m/z 348.9 (Fig. S26).

3. Supporting Figures and Table

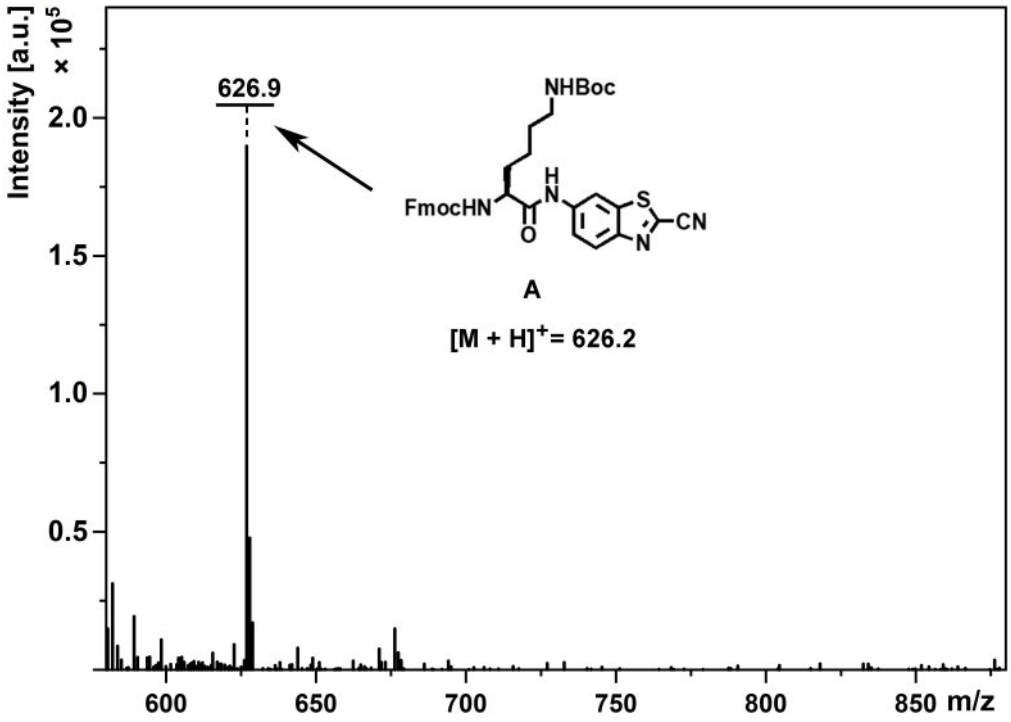


Fig. S1 ESI-MS spectrum of compound **A**.

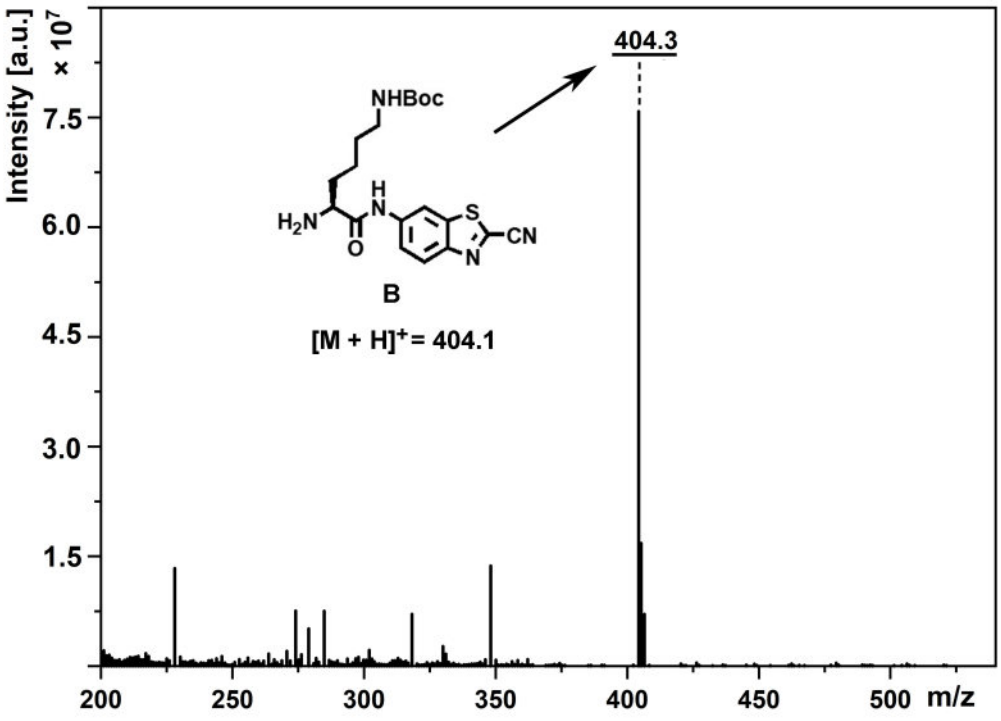


Fig. S2 ESI-MS spectrum of compound **B**.

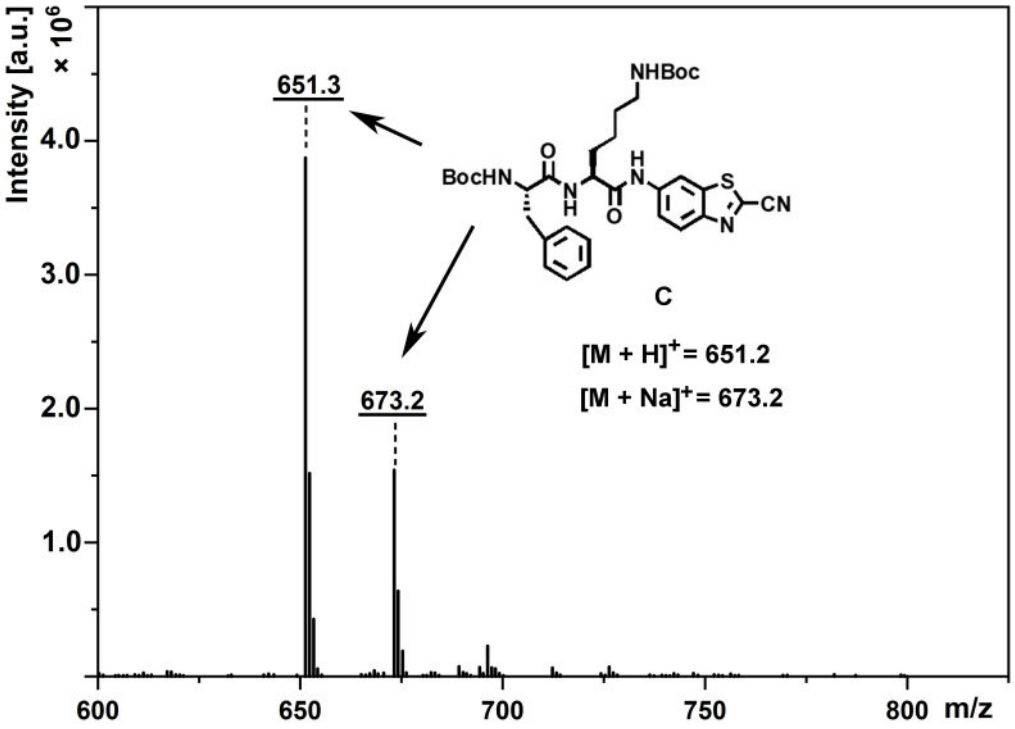


Fig. S3 ESI-MS spectrum of compound **C**.

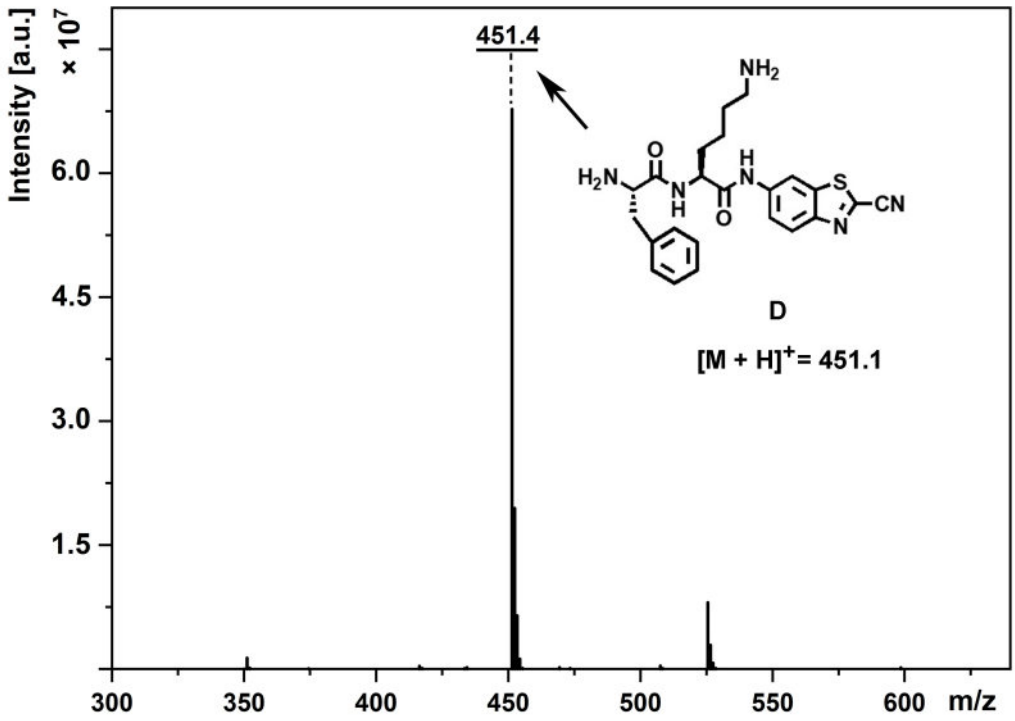


Fig. S4 ESI-MS spectrum of compound **D**.

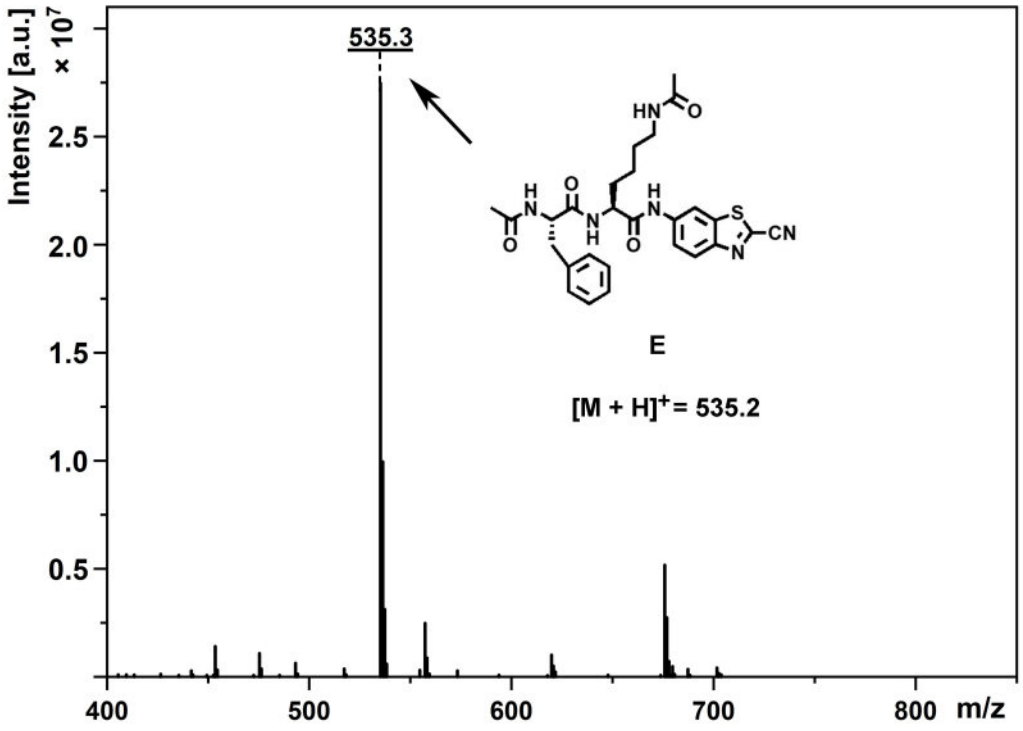


Fig. S5 ESI-MS spectrum of compound **E**.

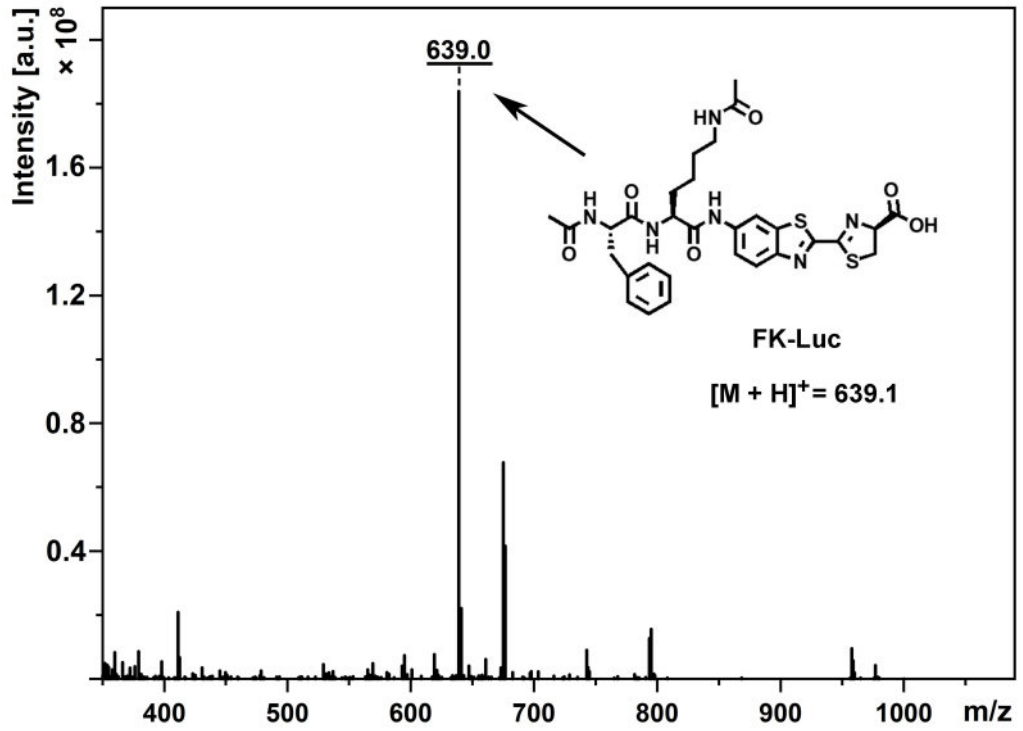


Fig. S6. ESI-MS spectrum of compound **FK-Luc**.

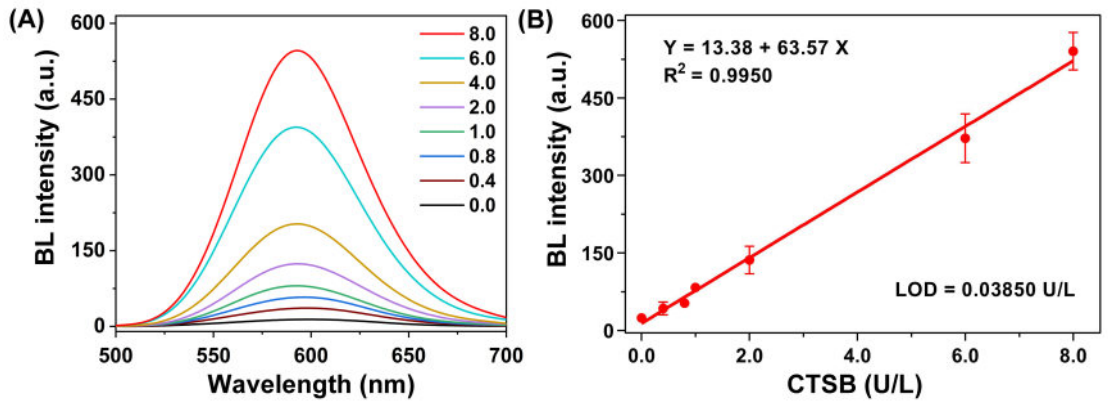


Fig. S7 (A) BL spectrum of **FK-Luc** incubated with different concentrations of CTSB at 37 °C for 2 h in working buffer (400 mM CH_3COONa, 4 mM EDTA, 2 mM GSH, pH = 7.0). Wavelength for detection: 500-700 nm. (B) Calibration curve of CTSB concentration as a function of BL intensity at 595 nm.

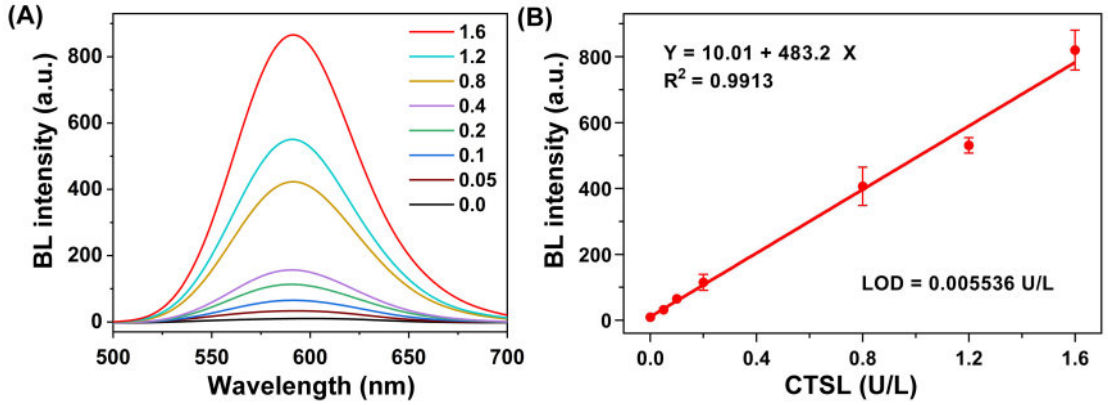


Fig. S8 (A) BL spectrum of 25 μM **FK-Luc** incubated with different concentrations of CTSL at 37 °C for 2 h in working buffer (400 mM CH_3COONa, 4 mM EDTA, 2 mM GSH, pH = 7.0). Wavelength for detection: 500-700 nm. (B) Calibration curve of CTSL concentration as a function of BL intensity at 595 nm.

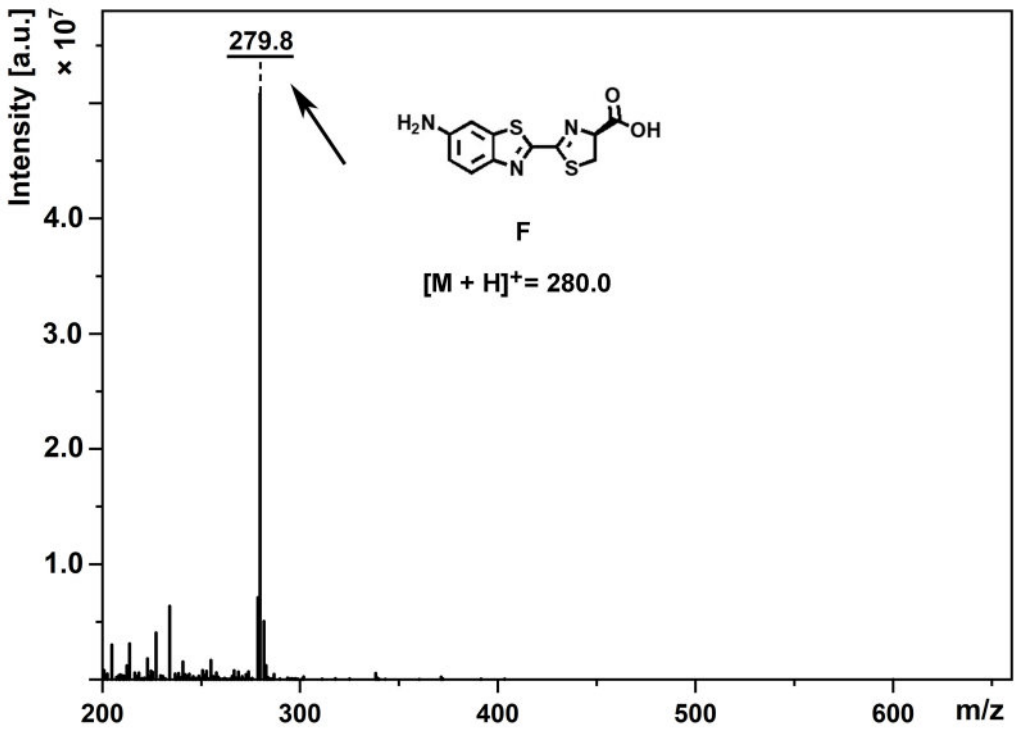


Fig. S9 ESI-MS spectrum of compound **F**.

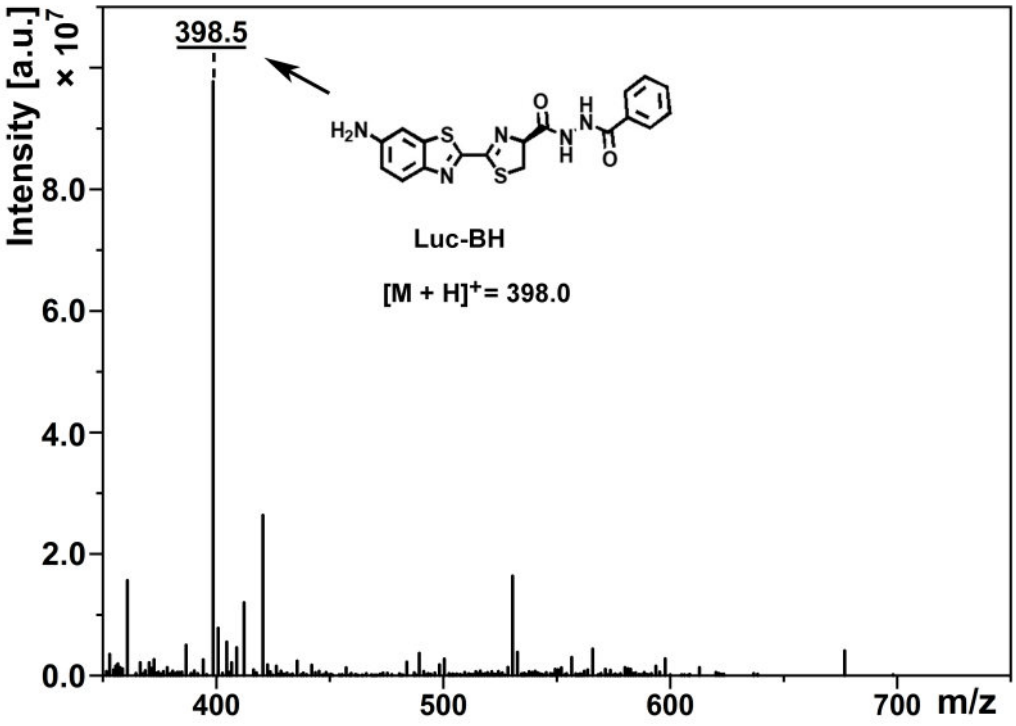


Fig. S10 ESI-MS spectrum of compound **Luc-BH**.

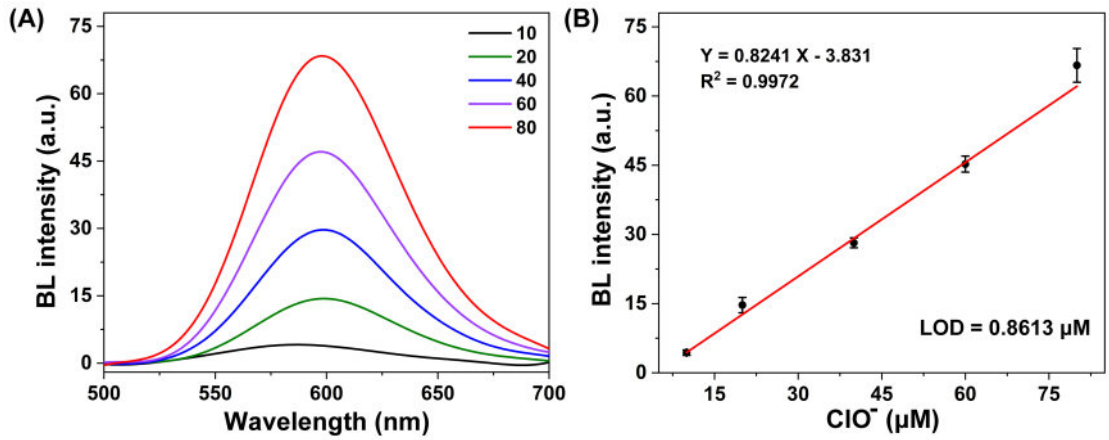


Fig. S11 (A) BL spectrum of 25 μM **Luc-BH** incubated with different concentrations of ClO^- at 37 °C for 2 h in the working buffer (400 mM CH_3COONa, 4 mM EDTA, 2 mM GSH, pH = 7.0). Wavelength for detection: 500-700 nm. (B) Calibration curve of ClO^- concentration as a function of BL intensity at 595 nm.

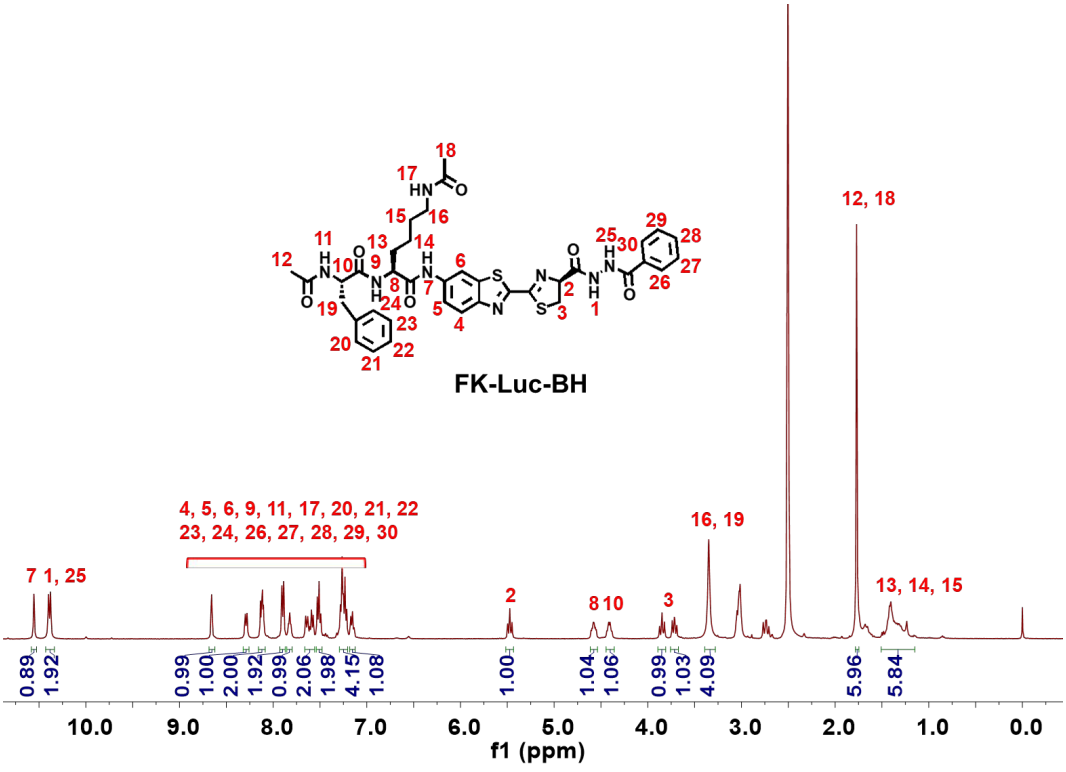


Fig. S12 ^1H NMR spectrum of **FK-Luc-BH** in DMSO-d_6.

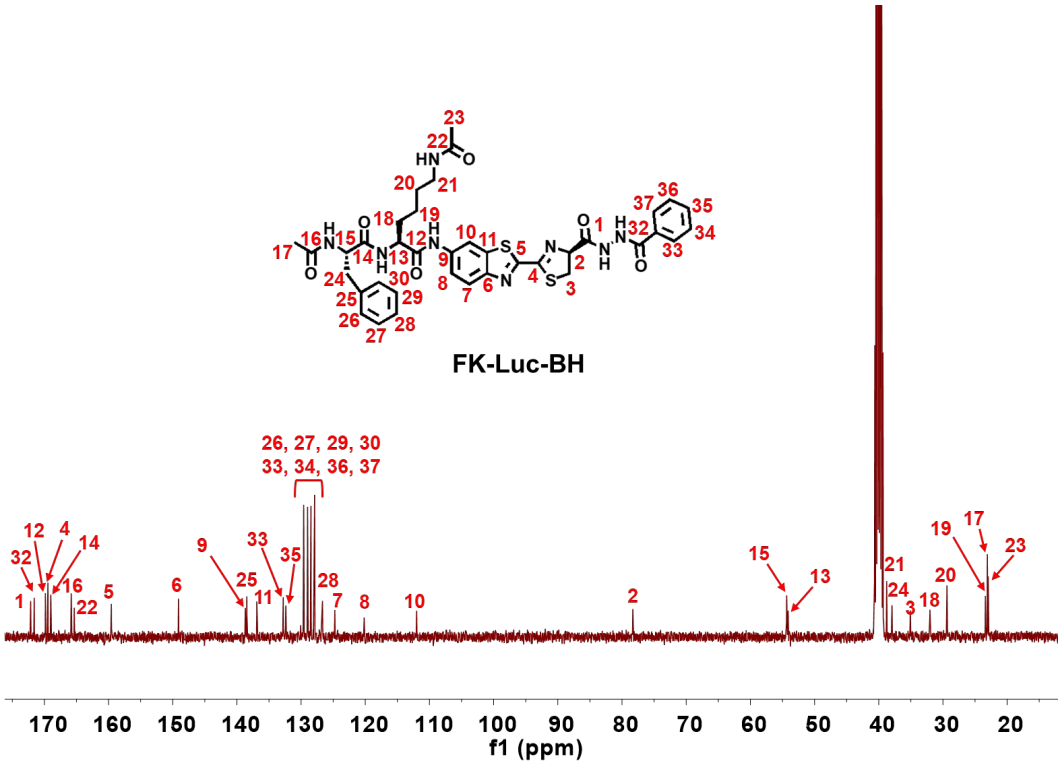


Fig. S13 ^{13}C NMR spectrum of **FK-Luc-BH** in DMSO-d_6.

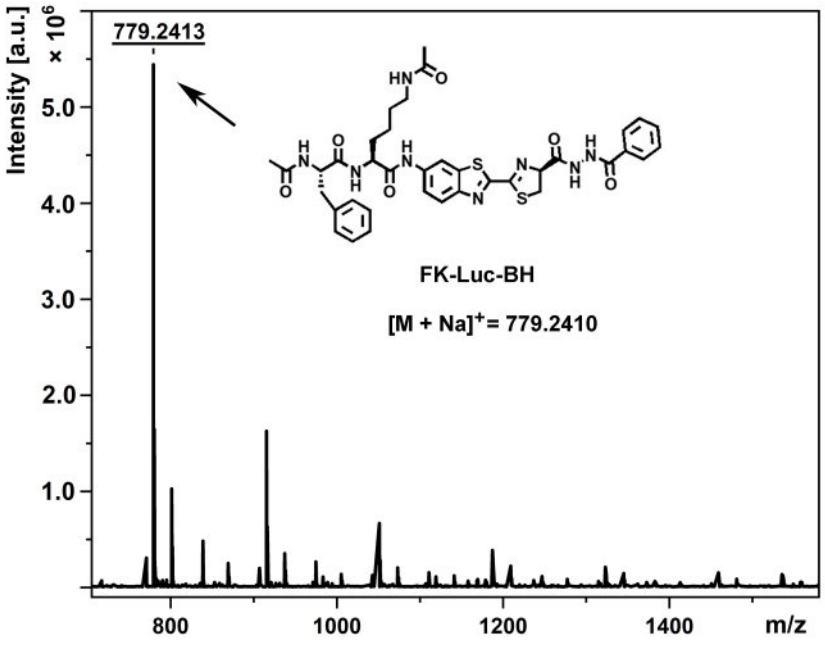


Fig. S14 HR-ESI-MS spectrum of compound **FK-Luc-BH**.

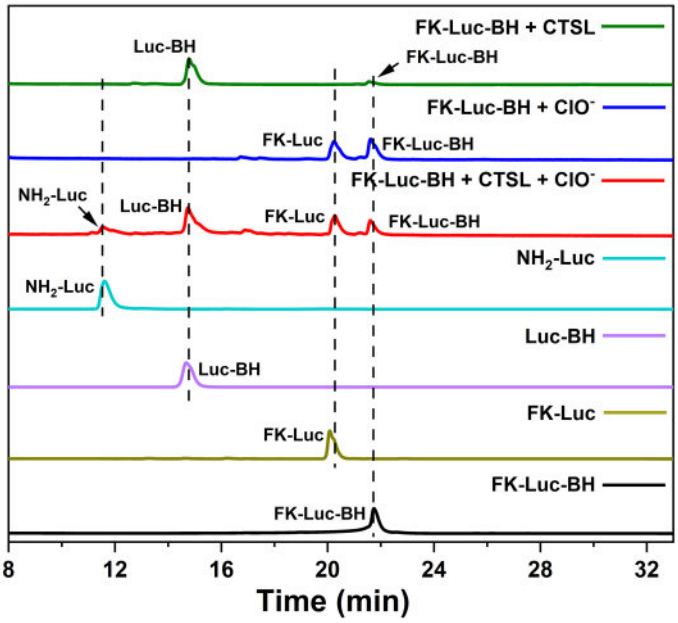


Fig. S15 HPLC trace of **NH_2-Luc** (cyan), **Luc-BH** (purple), **FK-Luc** (dark yellow), 25 μM **FK-Luc-BH** (blank) or 25 μM **FK-Luc-BH** in the presence 2 U/L CTSL (olive), 1 mM ClO^- (blue), 2 U/L CTSL together with 1 mM ClO^- (red) in CTSL working buffer (400 mM CH_3COONa, 4 mM EDTA, 2 mM GSH, 10 % DMSO, pH = 7.0) at 37 °C for 4 h.

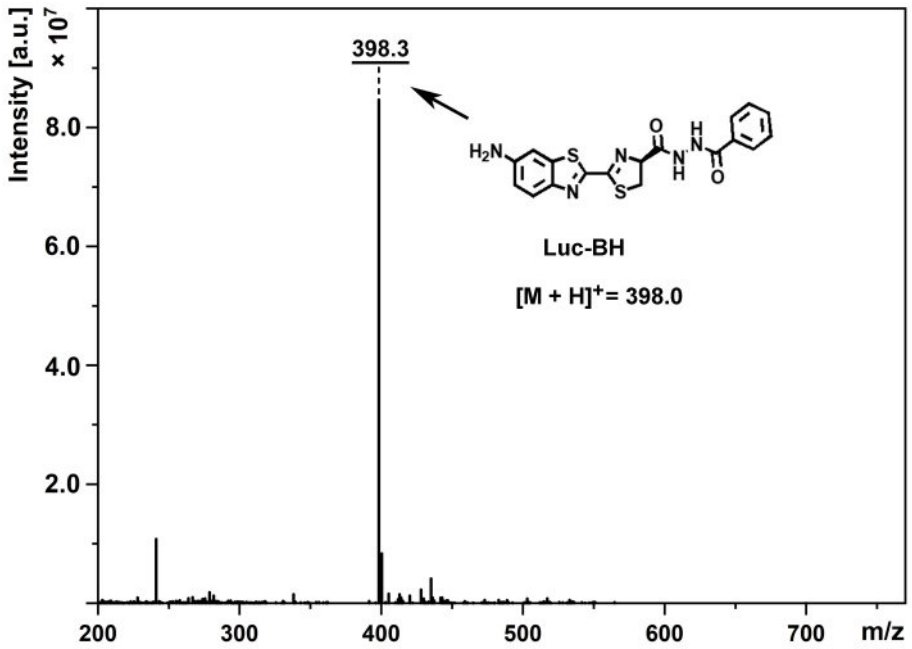


Fig. S16 ESI-MS spectrum of Fig. S15 at 14.5 min.

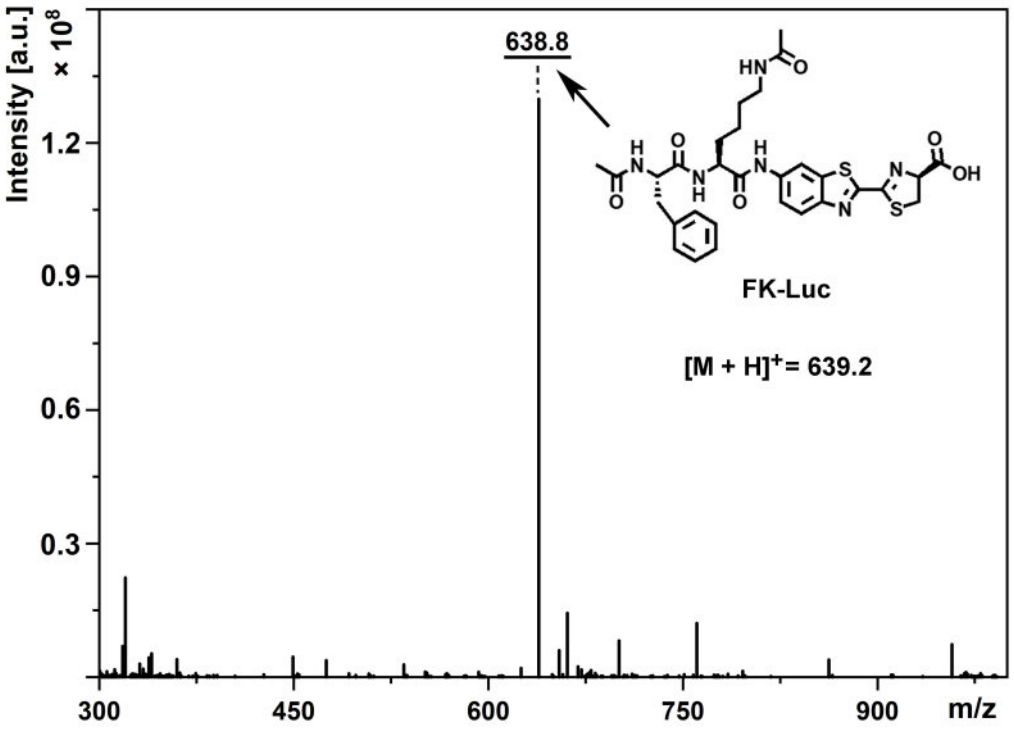


Fig. S17 ESI-MS spectrum of Fig. S15 at 20 min.

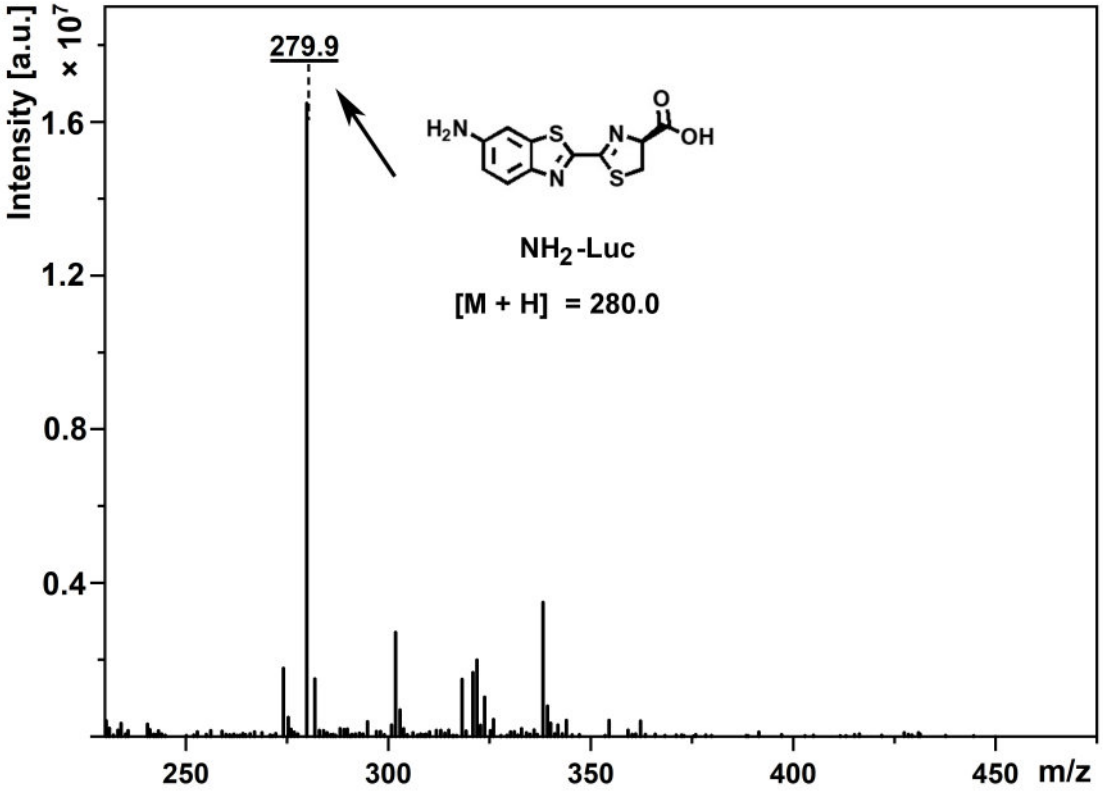


Fig. S18 ESI-MS spectrum of Fig. S15 at 11.5 min.

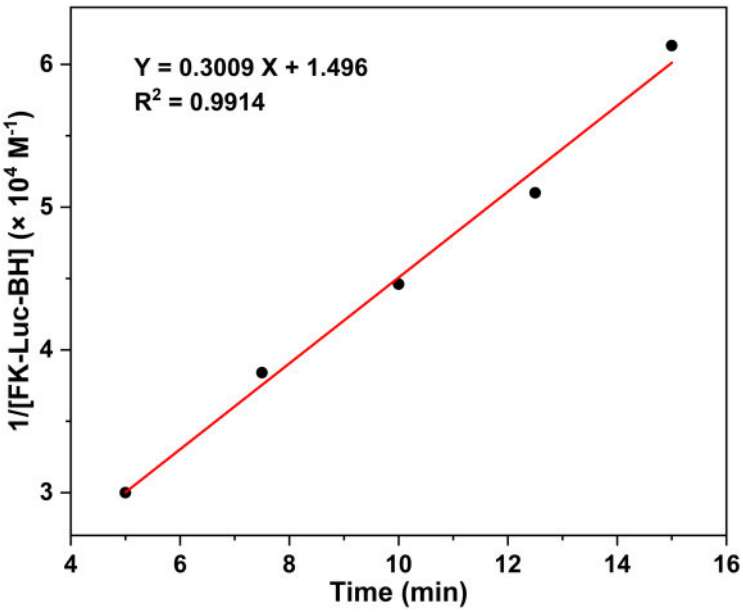


Fig. S19 Linear regression analysis of the reciprocal of the concentration of remaining **FK-Luc-BH** versus the reaction time. Conditions: 1 mM ClO^- and 50 μM **FK-Luc-BH** in CTSL working buffer without GSH (400 mM CH_3COONa, 4 mM EDTA, 10% DMSO, pH = 7.0).

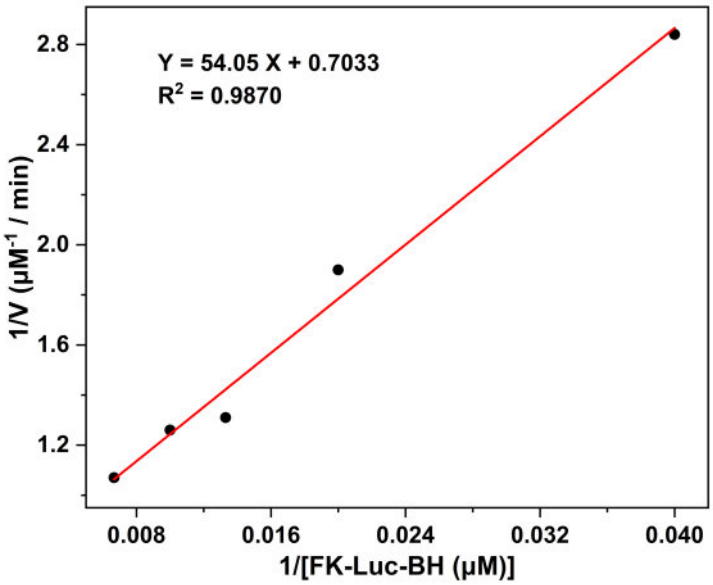


Fig. S20 Lineweaver-Burk plots of **FK-Luc-BH** (25-150 μM) catalyzed by 40 U/L CTSB.

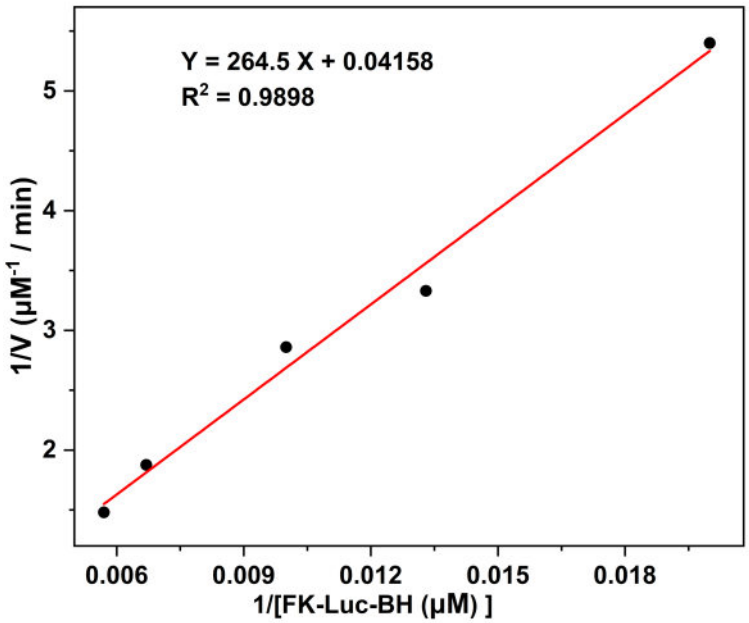


Fig. S21 Lineweaver-Burk plots of **FK-Luc-BH** (50-175 μM) catalyzed by 5 U/L CTSL.

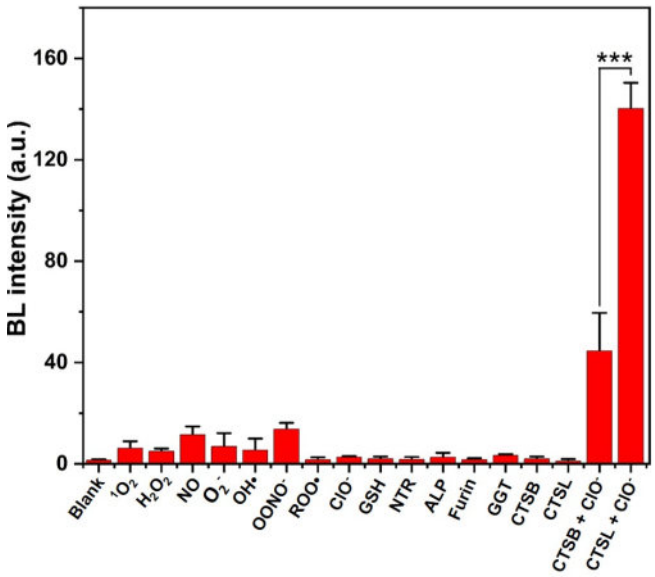


Fig. S22 BL responses of **FK-Luc-BH** to various biological species, including 200 μM $^1O_2$, 200 μM H_2O_2, 200 μM NO, 200 μM O_2^-, 200 μM •OH, 200 μM $ONOO^-$, 200 μM ROO•, 200 μM ClO^-, 1 mM GSH, 10 U/L NTR, 100 U/L ALP, 100 U/L GGT, 100 U/L furin, 2 U/L CTSB, 2 U/L CTSL, 2 U/L CTSB together with 200 μM ClO^-, and 2 U/L CTSL together with 200 μM ClO^-, respectively. Error bars represent the standard deviations of three independent experiments, *p*-value was obtained from t-tests using comparisons of two groups (*** for p = 0.0008).

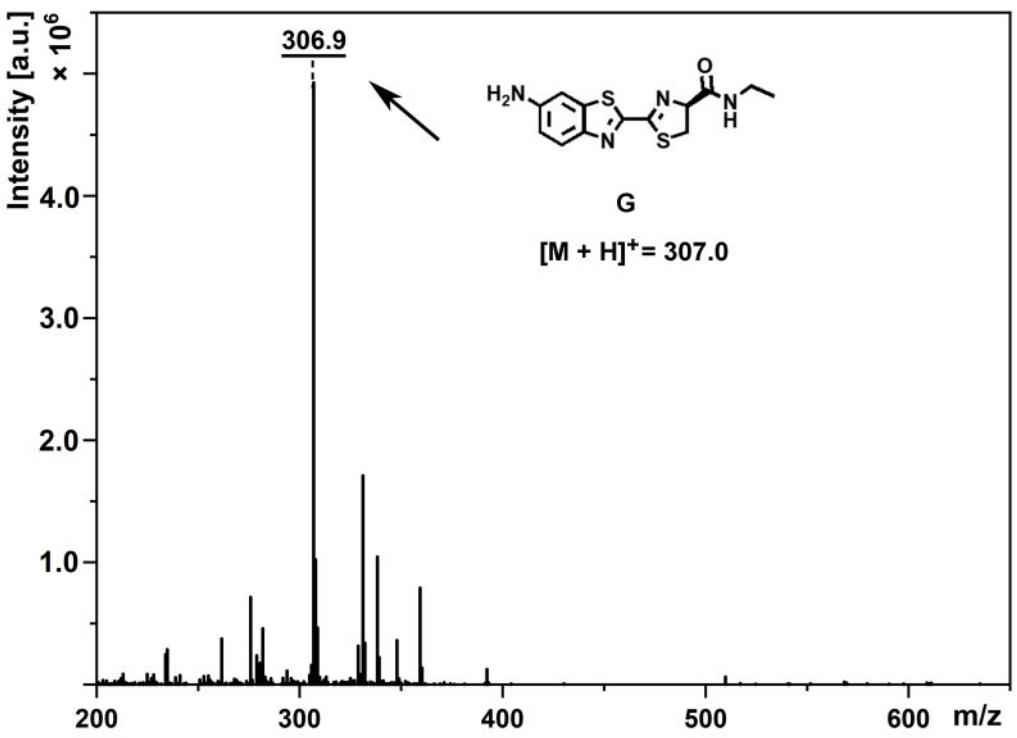


Fig. S23 ESI-MS spectrum of compound **G**.

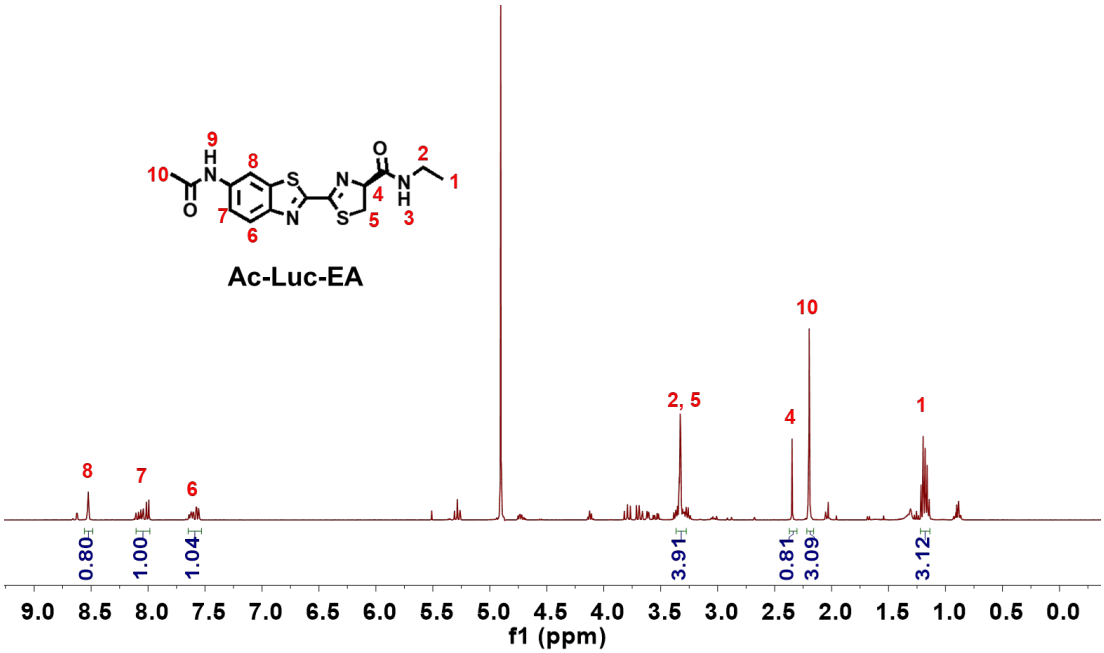


Fig. S24 ^{1}H NMR spectrum of **Ac-Luc-EA** in CD_3OD.

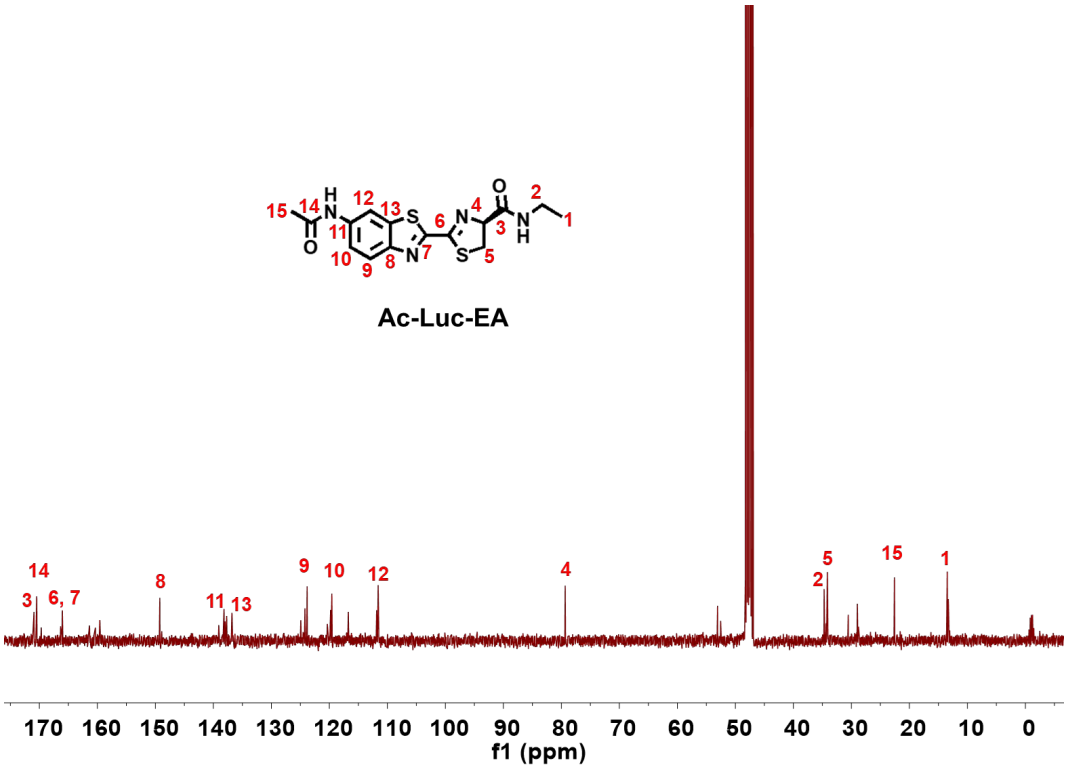


Fig. S25 ^{13}C NMR spectrum of **Ac-Luc-EA** in CD_3OD.

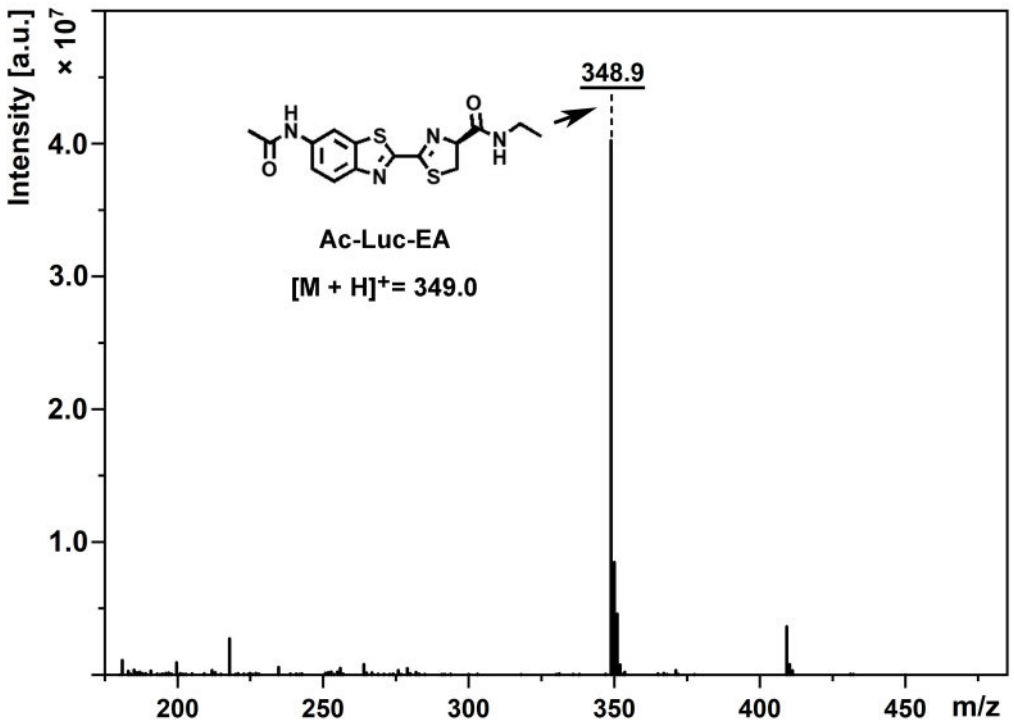


Fig. S26 ESI-MS spectrum of **Ac-Luc-EA**.

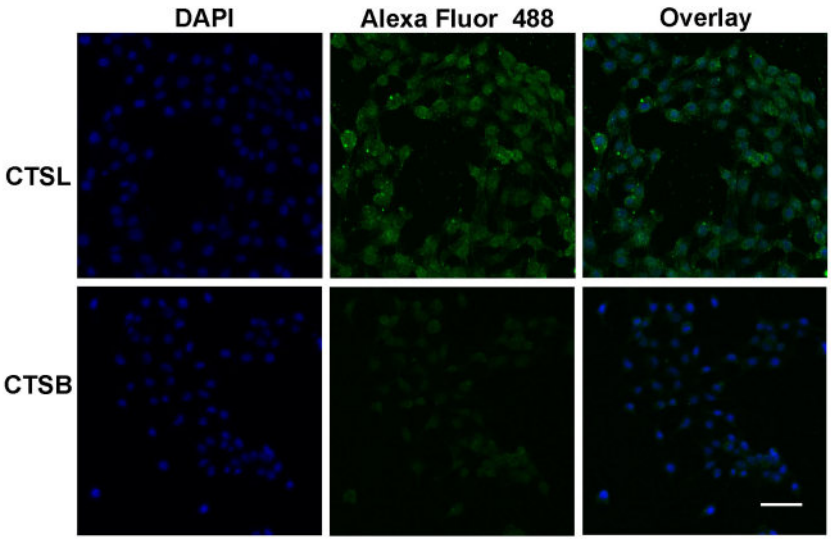


Fig. S27 Validation of CTSL and CTSB expression in 4T1-fLuc breast cancer cells using anti-CTSL or anti-CTSB immunofluorescent staining (green). Nuclei are counterstained with DAPI (blue). Scale bar: 100 μm.

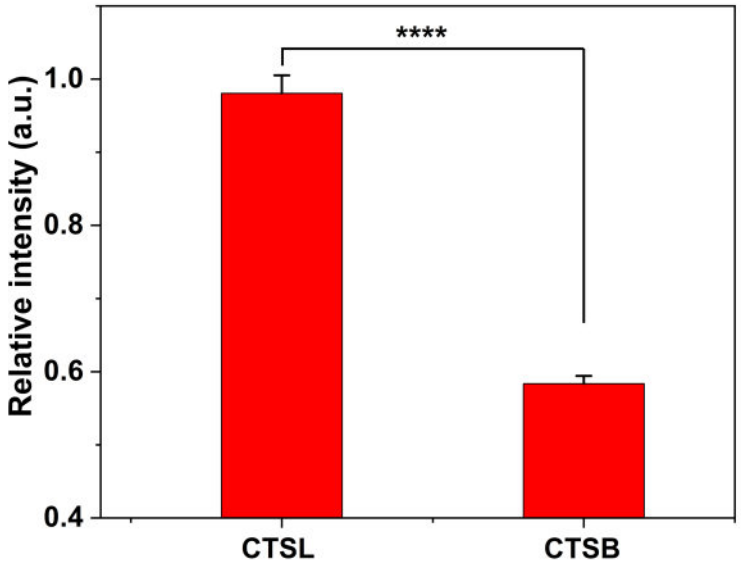


Fig. S28 Corresponding quantified intensity of fluorescence images in Fig. S27 using ImageJ software. Each error bar represents the standard deviation of three independent experiments, *p* value was obtained from t-tests using comparisons of two groups (**** for $p < 0.0001$).

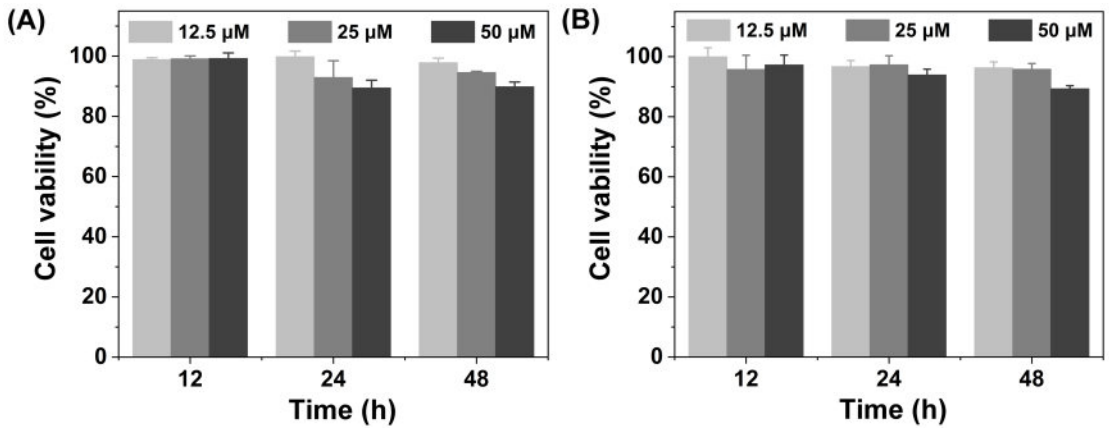


Fig. S29 MTT assay of **FK-Luc-BH** (A) and **Ac-Luc-EA** (B) on 4T1 cells (non-luciferase transfected), respectively. The error bar represents the standard deviation of three independent experiments.

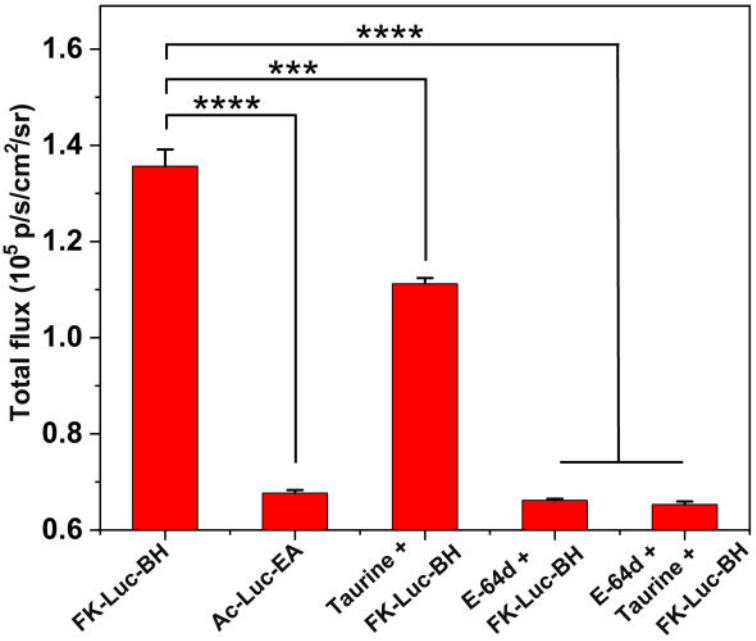


Fig. S30 Quantified total photons of Fig. 3B at 90 min. Each error bar represents the standard deviation of three independent experiments, *p*-value obtained from t-tests using comparisons of two groups (*** for $p = 0.0003$).

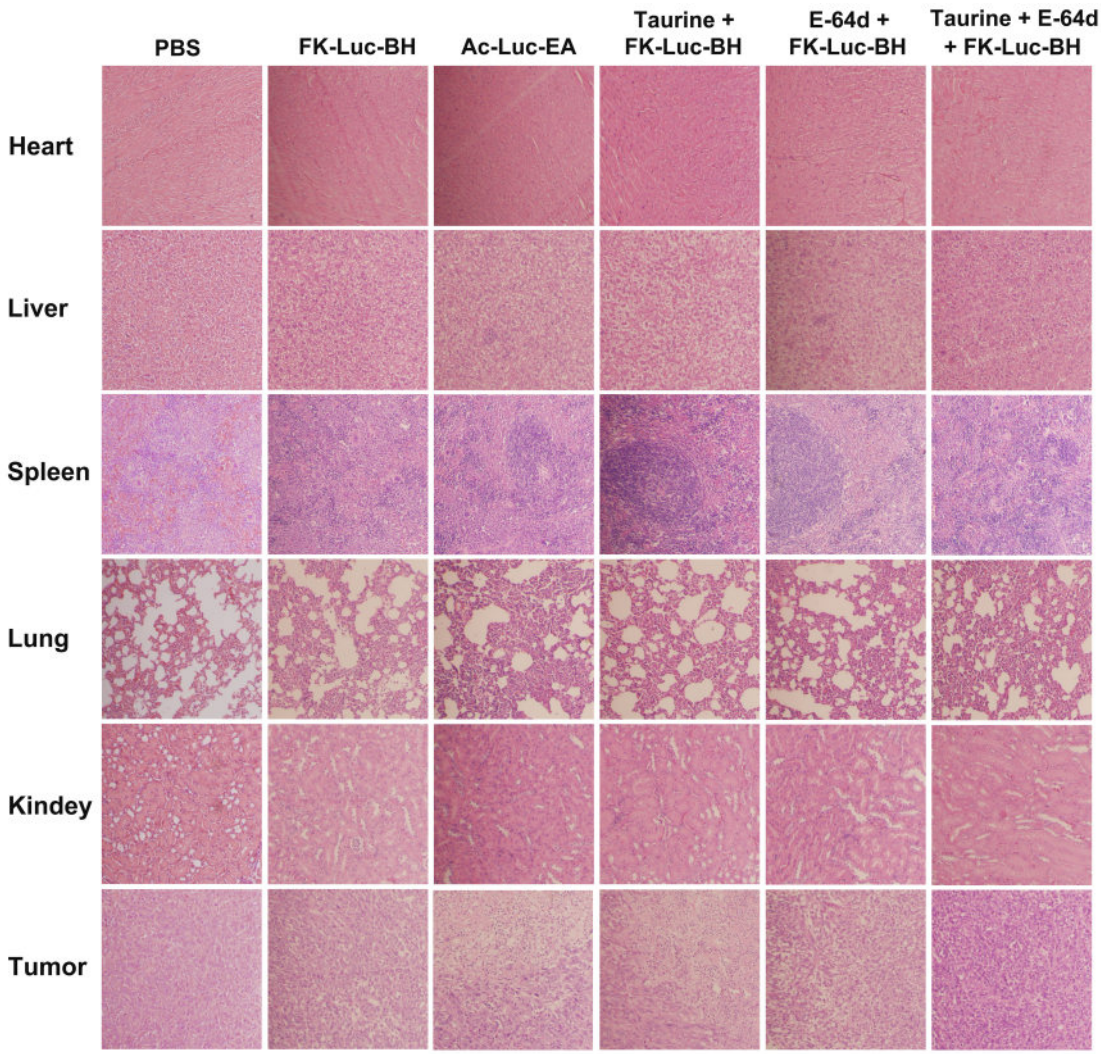


Fig. S31 H&E-stained images of the main organs and tumors collected after the last time point for BL imaging. Scale bar: 100 μm.

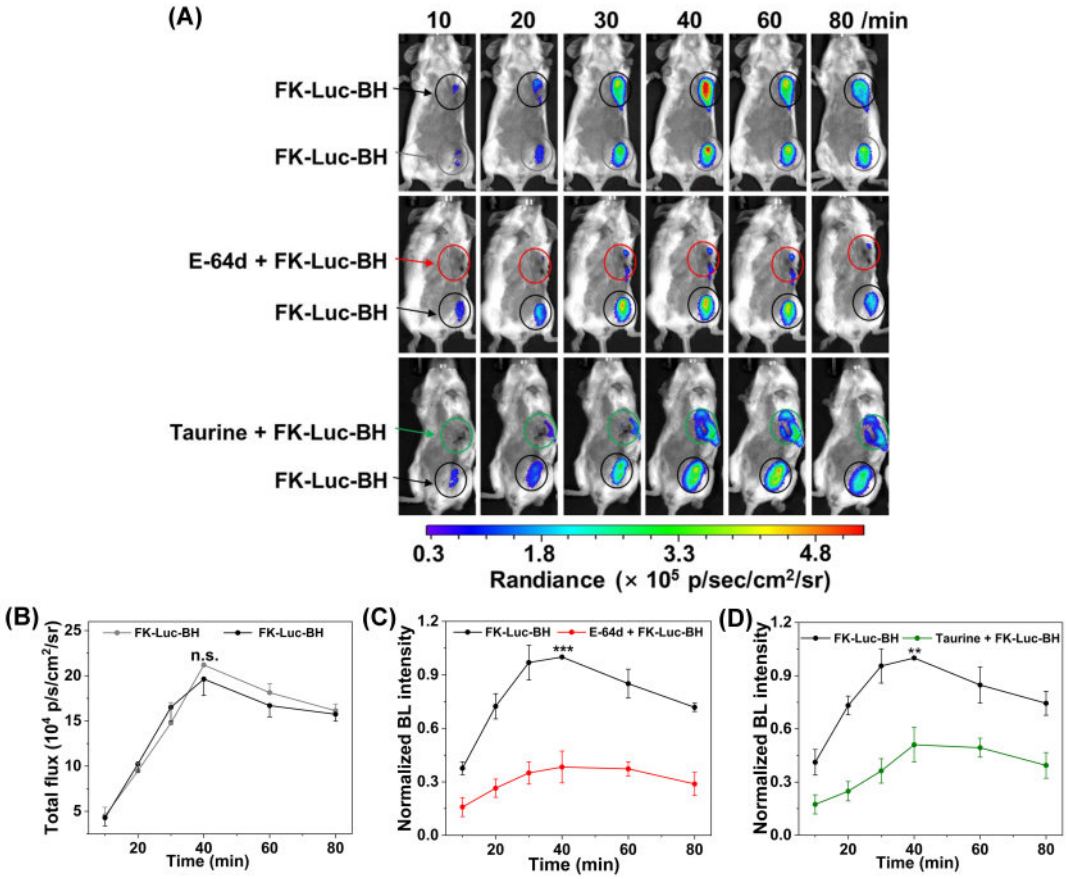


Fig. S32 (A) Time-course BLI of 4T1-fLuc tumor-bearing Balb/c mice after the intraperitoneal administration of 12.5 μmol/kg **FK-Luc-BH** without (the first row) and with the intratumoral pre-injection of 3 mmol/kg taurine for 5 min (the second row) or 0.05 mmol/kg E-64d for 5 min (the third row) into either of the tumors of each mouse. The images were acquired at 10, 20, 30, 40, 60, and 80 min. (B) Quantified total photon output of (A). Data are shown as mean ± s.d. for n = 3 mice, *p*-value obtained from t-tests using comparisons of two groups (n.s. for $p > 0.05$ in (B), ***for $p = 0.0009$ in (C), **for $p = 0.0091$ in (D)).

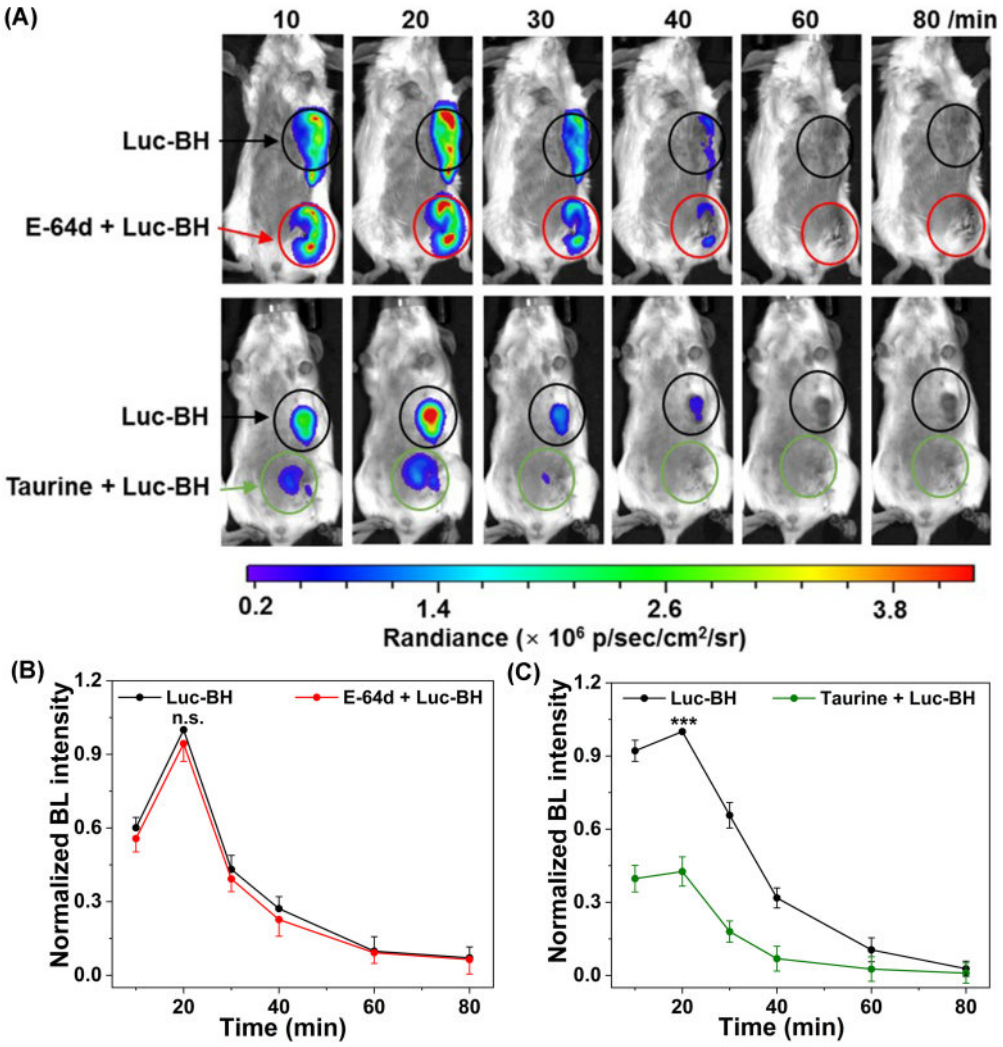


Fig. S33 (A) Time-course BLI of 4T1-fLuc tumor-bearing Balb/c mice after intratumoral pre-injection of 0.05 mmol/kg E-64d for 5 min (the first row) or 3 mmol/kg taurine for 5 min (the second row) into either of the tumors of each mouse, followed by intraperitoneal injection of 12.5 μmol/kg **Luc-BH**. The images were acquired at 10, 20, 30, 40, 60, and 80 min. (B) Quantified total photon output of (A). Data are shown as mean ± s.d. for n = 3 mice, *p*-value obtained from t-tests using comparisons of two groups (n.s. for $p > 0.05$ in (B), ***for $p = 0.0004$ in (C)).

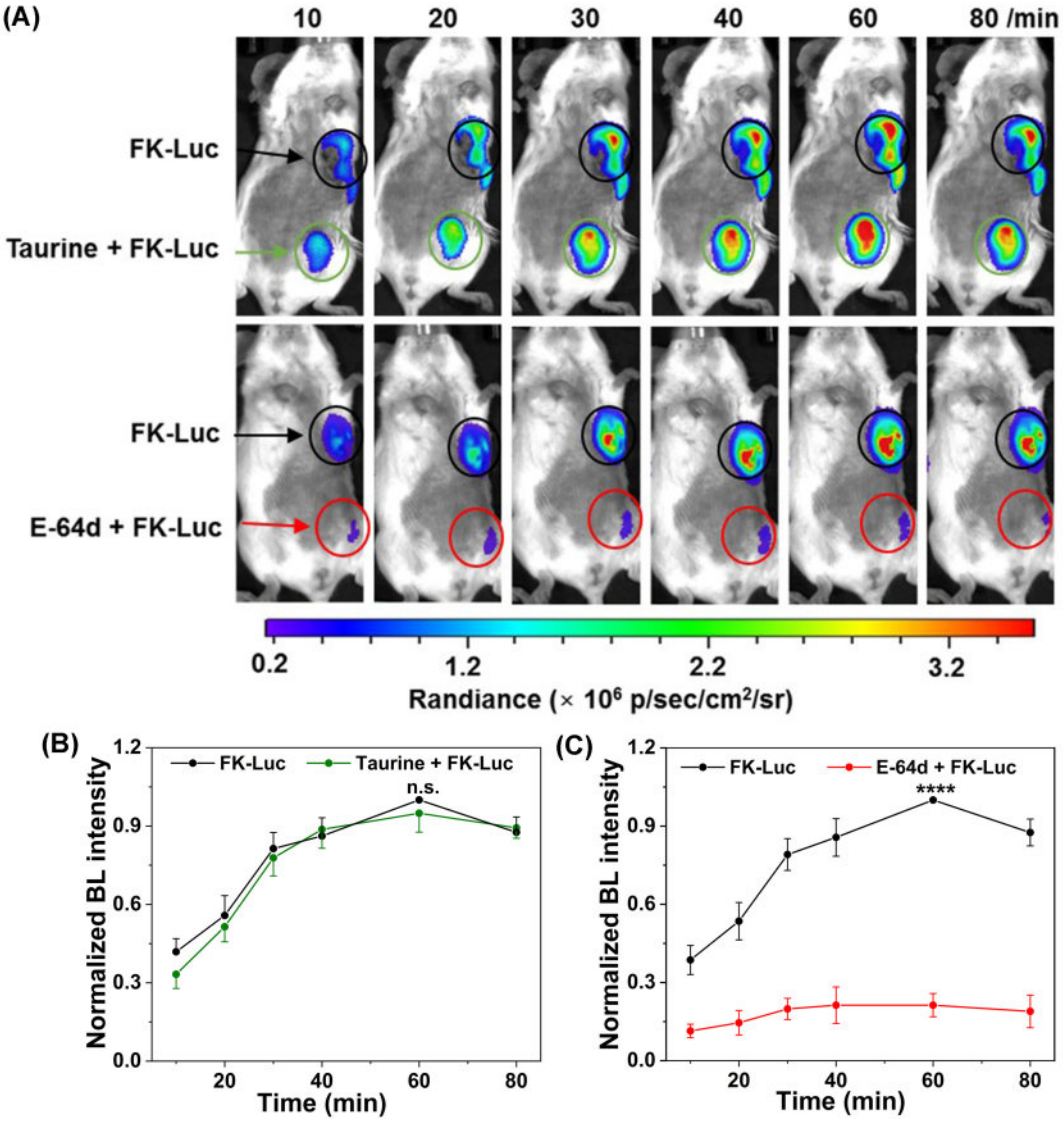


Fig. S34 (A) Time-course BLI of 4T1-fLuc tumor-bearing Balb/c mice after intratumoral pre-injection of 3 mmol/kg taurine for 5 min (the first row) or 0.05 mmol/kg E-64d for 5 min (the second row), followed by intraperitoneal injection of 12.5 μmol/kg **FK-Luc**. The images were acquired at 10, 20, 30, 40, 60, and 80 min. (B) Quantified total photon output of (A). Data are shown as mean ± s.d. for n = 3 mice, *p*-value obtained from t-tests using comparisons of two groups (n.s. for $p > 0.05$ in B, ****for $p < 0.0001$ in C).

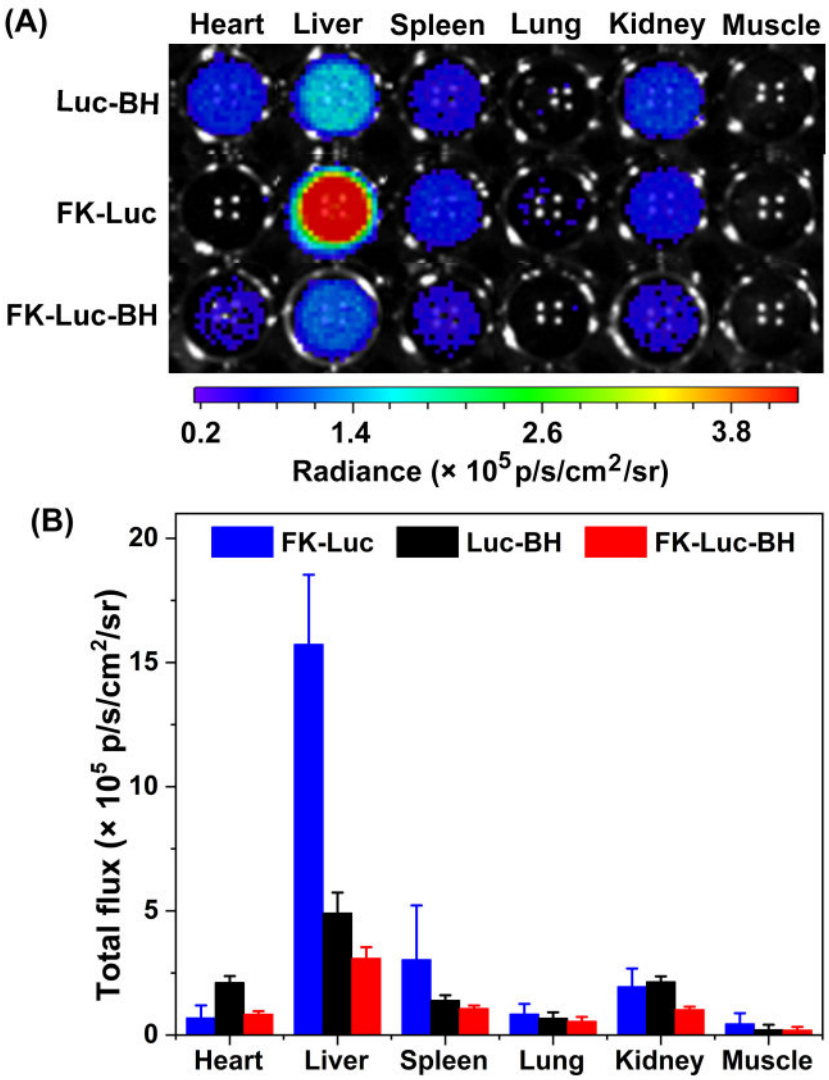


Fig. S35 (A) Ex vivo BL images of different tissue homogenates from Balb/c mice at 40 min after the intraperitoneal injection of 12.5 μmol/kg **FK-Luc-BH**, **FK-Luc**, and **Luc-BH**, respectively. (B) Quantified total photon output in A.

Table S1. HPLC condition of Fig. 2A and Fig. S15.

Time (min)	Flow (mL/min)	H_2O % (0.1% TFA)	CH_3CN % (0.1% TFA)
0	3.0	90	10
3	3.0	90	10
35	3.0	30	70
37	3.0	30	70
38	3.0	90	10
40	3.0	90	10

4. Reference

1 D. M. Mofford, S. T. Adams Jr, G. S. K. K. Reddy, G. R. Reddy, S. C. Miller, *J. Am. Chem. Soc.* 2015, **137**, 8684-8687.