Supplementary Information

**General Information**

**Materials**

All solvents and reagents used for solid phase peptide (SPPS) synthesis were purchased from commercial suppliers including GL Biochem (Shanghai, China), Energy Chemical (Shanghai, China), Hanhong Chemical (Shanghai, China) and Tenglong Logistics (Shenzhen, China) and were used without further purification unless otherwise stated.

All air and moisture sensitive reactions were carried out in flame-dried glassware under a nitrogen atmosphere. Reactive liquid compounds were measured and transferred by gas-tight syringes and were added in the reaction flask through rubber septa. Tetrahydrofuran (THF) were freshly distilled from sodium benzophenoneketyl. Toluene was distilled from CaH$_2$. Unless otherwise noted, all reagents were obtained commercially and used without further purification.

**NMR spectrum:**

$^1$H and $^{13}$C NMR spectra were collected on 500 MHz NMR spectrometers (Bruker AVANCE) using CDCl$_3$. Chemical shifts are reported in parts per million (ppm). Chemical shifts for protons are reported in parts per million downfield and are referenced to residual protium in the NMR solvent (CDCl$_3$ = δ 7.26). Chemical shifts for carbon are reported in parts per million downfield and are referenced to the carbon resonances of the solvent (CDCl$_3$ = δ 77.0). Data are represented as follows: chemical shift, multiplicity (br = broad, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet), coupling constants in Hertz (Hz), integration.

**Mass spectroscopy:**

Mass spectra were in general recorded on a QSTAR Elite (ABI).

**Chromatography:**

Column chromatography was performed with silica gel (200-300 mesh ASTM).

**Experimental Section**

**Peptide synthesis**

Peptides were synthesized on Rink-amide-MBHA resin using manual Fmoc/tBu solid phase peptide synthesis. Coupling was performed using HCTU/DIPEA for 3 h with N$_2$ bubbling. Coupling between PEG2000 and peptide was conducted using EDC/DIPEA for 5 h with N$_2$ bubbling in DCM. Cy5 labelling was performed in solution with the solution of Cy5-NHS ester in NaHCO$_3$ (10 mM, pH = 8.0) overnight. Final resins were treated with 95% (v/v) TFA/TIS/H$_2$O (95 : 2.5 : 2.5) for 2 h. After air removal of most of the TFA, products were triturated with hexane/Et$_2$O (1: 2), dissolved in CH$_3$CN/H$_2$O (1 : 1). Crude peptides were purified on RP-HPLC (Shimadzu (Kyoto, Japan), Agilent (Santa Clara, CA, USA) Zorbax SB-Aq: 4.6 × 250 mm, 220 and 254 nm) and confirmed by Shimadzu LC-MS 2020 mass spectrometer equipped with Agilent Zorbax SB-Aq column.

**Intramolecular disulfide bond formation**

Air oxidation: The solution containing the deprotected peptide at a dilute solution (lower than 0.2 mg/mL)
was adjusted to PH 8.0 by NH$_4$HCO$_3$. The reaction was slowly stirred for 24 h, and completely building of intramolecular disulfide bond was verified by HPLC and mass spectrometry.

**Cell Viability**

100 µL of 4 × 10$^4$/mL cell suspension was seeded in each well of the 96 well plate and allowed to grow in DMEM supplemented with 10% FBS supplied with 5% CO$_2$ overnight. Then, the cells were treated with serial dilution of compounds at 310K in 5% FBS containing media for 24 h. At the end of the compounds exposure, 20 µL of MTT reagent was added and incubated at 310K for 4 h. The absorbance of formazan product was measured at 490 nm by a microplate reader (Perkin Elmer, Envision, 2104 Multilabel Reader). Cells without peptide treatment were regarded as control.

**Confocal microscopy imaging**

MDA-MB-231 cells or HEK293T cells were seeded on coverslip at 310K in the presence of 5% CO$_2$. Then cells were then incubated with 22 µM FEB-2000, FEB-2000-iRGD or IR-783 for 6 h. After that, FEB derivatives and IR-783 containing media was removed followed by washing with phosphate buffered saline (PBS) twice. Then nuclei were stained with 4’,6-diamidino-2-phenylindole (DAPI) for 5 min then washed with PBS. The coverslips were mounted onto slides and visualized by confocal laser scanning microscope.

**Xenograft tumour model**

Four-week-old nude mice were purchased from the Vital River (Beijing, China). Ambient temperature was controlled at 293 to 295 K with 12 h light/12 h dark cycles. Approximately 1 × 10$^6$ MDA-MB-231 cells were injected into the mammary fat pads of female mice in a vehicle of 4 mg/mL Matrigel (BD). One week later, mice bearing tumours around 50 mm$^3$ in volume were randomly divided into groups. FEB-2000 and FEB2000-iRGD were injected intravenously at a dose of 270 µM, 100 µL. Cy-iRGD were injected intravenously at a dose of 50 µM, 100 µL. Mice were anesthetized with isoflurane and imaged using IVIS Spectrum Pre-clinical In Vivo Imaging System (535 nm/640 nm for FEB derivatives and 640 nm/680 nm for Cy5 derivatives). Fluorescence was calculated with Living Image. The values for each mouse were averaged.

The animal experiments complied to the Regulations of Guangdong Province on the Administration of Laboratory Animals, with the approval number of SIAT-IRB-160128-YYS-LZG-A0164.

**Spectral characterizations of FEB derivatives.**

UV-Vis-NIR spectrophotometer (Cary 6000i) with background correction was employed to measure the optical absorption spectrum of FEB derivatives in the range of 300-1,200 nm. A home build setup was used to measure the fluorescence spectrum in the region of 500-900 nm using an array detector (Princeton OMA-V) and a spectrometer (Acton SP2300i) under a 535-nm diode laser (RMPC lasers) excitation. The obtained emission spectra were further corrected by the detector sensitivity profile and the absorbance features of the filter.

**Quantum yield measurement**

Determination of fluorescence quantum yield of FEB2000 and FEB2000-iRGD. Fluorescence quantum yield of these dyes was measured in water according to a previous reported method. The commercial
fluorescent dye RHB was used as the reference sample with the quantum yield of 31%. RHB was dissolved in water, and diluted to different concentration with absorbance value at 500 nm of below 0.1, using a ultraviolet-visible- near-infrared absorbance spectrometer (Cary 600i). The fluorescence spectra in the range of 500- to 800-nm were collected under the 500 nm diode laser (RMPC lasers) excitation. The absorption and emission of FEB-2000 and FEB2000-iRGD in water were measured using the same method. The integrated fluorescence intensity was plotted against absorbance at the excitation wavelength of 500 nm and fitted into a linear function. The slope of FEB derivatives were compared with dye RHB, and the quantum yields were determined by following equation.

\[ QY_{FEB} = QY_{RHB} \cdot \frac{slope_{FEB}}{slope_{RHB}} \cdot \left( \frac{n_{FEB}}{n_{RHB}} \right)^2 \]

**In vivo toxicity**

FEB2000 and FEB2000-iRGD (270 μM, 100 μL) was injected intravenously into nude mice. All collected tissue sections (4 μm) were stained with hematoxilin and eosin (H&E) and subsequently processed for histopathological examination under light microscope.
Schemes and Figures

Fig. S1 Absorption coefficient: FEB-2000 = 28530 M/cm, FEB-2000-iRGD = 27300 M/cm.

![Absorption Coefficiency](image)

Fig. S2 Fluorescence intensity of FEB-2000 (Left) and FEB-2000-iRGD (Right) in water, PBS, and FBS measured over 1 week.

![Fluorescence Intensity](image)

Fig. S3 Quantum yield measurement of molecular fluorophore FEB2000, FEB2000-iRGD, RHB.
Integrated NIR-II fluorescence intensity plotted as a function of absorbance at 500 nm for dye solutions.
The data was fitted into a linear function. Slope_{RHB}=3.228 \times 10^6, Slope_{FEB-2000}=3.649 \times 10^6, Slope_{FEB-2000-iRGD}=3.802 \times 10^6.

![Quantum Yield](image)
**Fig. S4** Confocal laser-scanning microscopy images of MDA-MB-231 cells (a) and HEK 293T cells (b) incubated with PBS, FEB-2000, FEB-2000-iRGD and IR-783 at 310 K for 6 h. Scale bar = 5 μm.

**Fig. S5** a. Confocal laser-scanning microscopy images of MDA-MB-231 cells incubated with PBS, FEB-2000-iRGD at 310 K for 6 h. Scale bar = 20 μm; b. Cytotoxicity of FEB-2000-iRGD on MDA-MB-231 cell line and HeLa cell line.

**Fig. S6** Fluorescence intensity of organ and tumour samples 48 h post-injection.
**Scheme S1:** Synthesis of FEB.

1. Synthesis of 5-(9H-fluoren-2-yl)-2,3-dihydrothieno[3,4-b][1,4]dioxine (3).

   ![Diagram of Scheme S1](image)

   2-bromo-9H-fluorene 1 (5.0 g, 20.4 mmol) and tributyl(2,3-dihydrothieno[3,4-b][1,4]dioxin-5-yl)stannane 2 (9.2 g, 21.4 mmol) were dissolved in 40 mL toluene under protective gas atmosphere then Pd(PPh₃)₄ (200 mg) was added. After reflux 6 h, the crude product was subjected to column chromatography on silica gel to afford 3 as a light yellow solid (5.8 g, 94%).

   - **¹H NMR** (500 MHz, Chloroform-d) δ 7.94 (dd, J = 3.8, 1.7 Hz, 1H), 7.82 – 7.74 (m, 3H), 7.56 (dd, J = 7.3, 2.0 Hz, 1H), 7.40 (td, J = 7.5, 2.7 Hz, 1H), 7.36 – 7.29 (m, 1H), 6.41 – 6.26 (m, 1H), 4.37 – 4.31 (m, 2H), 4.29 – 4.23 (m, 2H), 3.95 (s, 2H).
   - **¹³C NMR** (126 MHz, CDCl₃) δ 37.60, 65.10, 65.41, 98.00, 118.68, 120.45, 120.62, 123.15, 125.43, 125.64, 127.22, 127.41, 132.37, 138.60, 140.84, 142.11, 144.91, 144.05, 144.30. HRMS(ESI) calcd for C₁₉H₁₅O₂S⁺, ([M+H⁺]) 307.0793, Found 307.0787.

2. Synthesis of 5-(9,9-bis(6-bromohexyl)-9H-fluoren-2-yl)-2,3-dihydrothieno[3,4-b][1,4]dioxine (4).

   5-(9H-fluoren-2-yl)-2,3-dihydrothieno[3,4-b][1,4]dioxine 3 (3.0 g, 9.8 mmol) and 1,6-dibromohexane (9.7 g, 40 mmol) were dissolved in 50 mL THF at 0 °C. Then potassium tert-butanolate (2.5 g, 2.1 mmol) which dissolved in THF was added dropwise. After 6 h at rt, the crude product was subjected to column chromatography on silica gel to afford 4 as a light yellow oil (4.9 g, 81%).

   - **¹H NMR** (500 MHz, Chloroform-d) δ 7.77 (dd, J = 8.0, 1.6 Hz, 1H), 7.71 (ddd, J = 7.9, 4.6, 0.8 Hz, 2H), 7.69 – 7.64 (m, 1H), 7.41 – 7.29 (m, 3H), 6.35 (s, 1H), 4.42 – 4.34 (m, 2H), 4.34 – 4.26 (m, 2H), 3.30 (t, J = 6.8 Hz, 4H), 2.02 (dt, J = 11.1, 5.7 Hz, 4H), 1.74 – 1.59 (m, 4H), 1.29 – 1.17 (m, 4H), 1.16 – 1.04 (m, 4H), 0.78 – 0.56 (m, 4H).
   - **¹³C NMR** (126 MHz, CDCl₃) δ 23.54, 27.76, 29.06, 32.65, 34.07, 40.20, 55.01, 64.52, 64.88, 77.29, 97.35, 118.20, 119.70, 119.88, 120.23, 122.77, 124.98, 126.92, 127.02, 130.02, 130.06, 139.69, 140.86, 142.38, 150.55, 150.77. HRMS(ESI) calcd for C₃₃H₃₇Br₂O₂S⁺, ([M+H⁺]) 630.0803, Found 631.0861.

3. Synthesis of FEB.
To a solution of Compound 4 2.0 g (3.18 mmol) in 25 mL THF at -78 °C under protection gas atmosphere, n-BuLi (1.6 M in Hexane, 2.4 mL, 3.8 mmol) was added dropwise. After the mixture was stirred at this temperature for another 1.5 h, tributyltin chloride (1.3 g, 3.8 mmol) was added to the solution. Then slowly warmed to room temperature and stirred for 8 h. After that the mixture was poured into water and extracted twice with ethyl acetate, the combined organic phase was dried with MgSO₄ and evaporated in vacuo without further purification. To a solution of compounds of a 195 mg (0.5 mmol), the crude product 1.4 g (1.5 mmol) in 15 mL toluene under protection gas atmosphere then Pd(PPh₃)₂Cl₂ 100 mg was added. The mixture was stirred at 110 °C for 12 h. After cooling to room temperature, the mixture was poured into water and extracted twice with ethyl acetate, dried with MgSO₄ and evaporated in vacuo. The crude product was subjected to column chromatography on silica gel and afforded FEB as a crimson solid (435 mg, 60 %).

1H NMR (500 MHz, Chloroform-d) δ 8.51 (s, 2H), 7.95 (d, J = 8.0 Hz, 2H), 7.80 (s, 2H), 7.74 (dd, J = 7.7, 5.0 Hz, 4H), 7.35 (dd, J = 12.7, 5.9 Hz, 6H), 4.51 (d, J = 4.3 Hz, 8H), 3.31 (t, J = 6.8 Hz, 8H), 2.14 – 1.97 (m, 8H), 1.69 (dt, J = 14.1, 6.9 Hz, 11H), 1.39 (dd, J = 14.7, 7.5 Hz, 5H), 1.32 – 1.19 (m, 13H), 1.13 (dd, J = 15.5, 7.3 Hz, 9H), 0.77 – 0.56 (m, 8H).

13C NMR (126 MHz, CDCl₃) δ 152.44, 150.81, 150.61, 141.08, 140.88, 140.03, 138.13, 131.93, 127.10, 126.94, 126.74, 125.49, 123.38, 122.77, 120.43, 120.34, 119.89, 119.74, 111.78, 77.31, 77.06, 76.80, 64.90, 64.57, 55.12, 40.23, 34.11, 32.65, 29.73, 29.05, 28.30, 27.78, 26.81, 23.55, 17.32, 13.65.

HRMS (ESI) calcd for C₆₈H₇₃Br₄N₂O₄S₃⁺, ([M+H⁺]) 1393.1466, Found 1393.1461.


Compound FEB 100 mg (0.069 mmol) was dissolved in 10 mL DMF and sodium azide 47 mg (0.72 mmol) and heated for 3 h at 70 °C, then added just as much water as to dissolve all solids. Then it was extracted twice with ethyl acetate, the combined organic phase was dried with MgSO₄ and evaporated in vacuo. The crude product was subjected to column chromatography on silica gel to afford dark crimson solid 95 mg (quant). The dark crimson solid was dissolved in 5 mL THF and CuTc10 mg, w-alkynyl-PEG-hydroxyl (average molecular weight was 2000) 560 mg, and TBTA (10 mg) was added. The system was stirred at rt for 0.5 h. Then filtered with diatomite, and the solution was evaporated in vacuo. When all the organic solvent was removed, the solid was dissolved in 15 mL water. Then transfer the water solution to the dialyzer (MWCO = 100KD). Then freeze out the water solution, FEB-2000 (250 mg) was afforded as a crimson solid. GPC results proved the four azide groups were all substituted.

(5) Synthesis of FEB-2000-iRGD
Compound **FEB** 100 mg (0.069 mmol) was dissolved in 10 mL DMF and sodium azide 47 mg (0.72 mmol) and heated for 3 h at 70 °C, then added just as much water as to dissolve all solids. Then it was extracted twice with ethyl acetate, the combined organic phase was dried with MgSO₄ and evaporated in vacuo. The crude product was subjected to column chromatography on silica gel to afford dark crimson solid 95 mg (quant). The dark crimson solid was dissolved in 5 mL THF and CuTc10 mg, w-alkynyl-PEG-iRGD (average molecular weight was 3000) 840 mg, and TBTA (10 mg) was added. The system was stirred at rt for 0.5 h. Then filtered with diatomite, and the solution was evaporated in vacuo. When all the organic solvent was removed, the solid was dissolved in 15 mL water. Then transfer the water solution to the dialyzer (MWCO = 100KD). Then freeze out the water solution, **FEB-2000-iRGD** was afforded as a crimson solid.

**Appendix**

**Mass spectra data for peptides.**

**RGD:** H-βAla-**CRGDRGPDC**-NH₂

**iRGD:** H-βAla-c(**CRGDRGPDC**)-NH₂

**Cy5-iRGD:** Cy5-βAla-c(**CRGDRGPDC**)-NH₂
MALDI-TOF-MS data

PEG2000-RGD:
PEG2000-IRGD:

AB Sciex TOF/TOF™ Series Explorer™ 2109

AB Sciex TOF/TOF™ Series Explorer™ 2109

S13
GPC data

FEB-N$_3$: 

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Conventional Calibration - Homopolymers: Results

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Annotations

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- Calculation Operator: admin / Administrator
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Conventional Calibration - Homopolymers: Results

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**Annotation**

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