*Electronic Supporting Information*

# An AI-Engineered Peptidyl Fluorescence Probe for GRP78: Advancing Renal Cell Carcinoma Diagnosis

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## 1. Experimental Section

**Materials.** All chemicals and solvents were purchased as reagent grade and used without further purification unless otherwise noted. Fetal bovine serum and penicillin/streptomycin were obtained from Biological Industries and Beijing Solarbio Science&Technoiogy Co., Ltd. Human GRP78 protein was purchased from Abcam. Fetal bovine serum and penicillin/streptomycin were obtained from Biological Industries and Beijing Solarbio Science&Technoiogy Co., Ltd. respectively. Roswell Park Memorial Institute 1640 (RPMI 1640), Dulbecco's Modified Eagle Medium (DMEM) and Modified Eagle Medium (MEM) were purchased from Beijing Solarbio Science&Technoiogy Co., Ltd.

**Instruments.** $^{1}H$ NMR and $^{13}C$ NMR spectra were recorded on the Bruker Avance-600 MHz and 151 MHz NMR spectrometers, respectively. ESI mass spectrometry was carried out on the AB Triple TOF 5600plus System. UV-visible spectra and steady-state emission experiments were performed on a Hitachi U-3900 spectrometer and a Hitachi F-7000 spectrometer, respectively. Cell and tissue imaging experiments were measured by Zeiss LSM880 Airyscan confocal laser scanning microscope. *In vivo* imaging assays were performed in the Perkin Elmer Lumina LT small animal optical imaging system.

**Cell culture.** Human colon carcinoma HCT116 cells were cultured and maintained in RPMI 1640 medium (supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin) at 37 °C and 5% $CO_2$ in a humidified incubator. Human embryonic kidney 293T cells, human breast cancer MCF-7 cells, mouse hepatoma Hepa1-6 cells, human cervical cancer Hela cells were cultured in DMEM medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Human renal adenocarcinoma ACHN cells were cultured in MEM medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. All cells were provided by the Institute of Biochemistry and Cell Biology, SIBS, CAS (China).

**Confocal laser scanning microscope (CLSM) imaging.** Cells were cultured overnight 14 mm glass coverslips overnight and washed with PBS (3 times × 2 mL).

After incubation with 10 μM probes for 120 min at 37℃, cells were washed with PBS (3 times × 2 mL) and subjected to CLSM imaging. The green channel (493-593 nm) was used to collect the probes signal under 488 nm as the excitation wavelength.

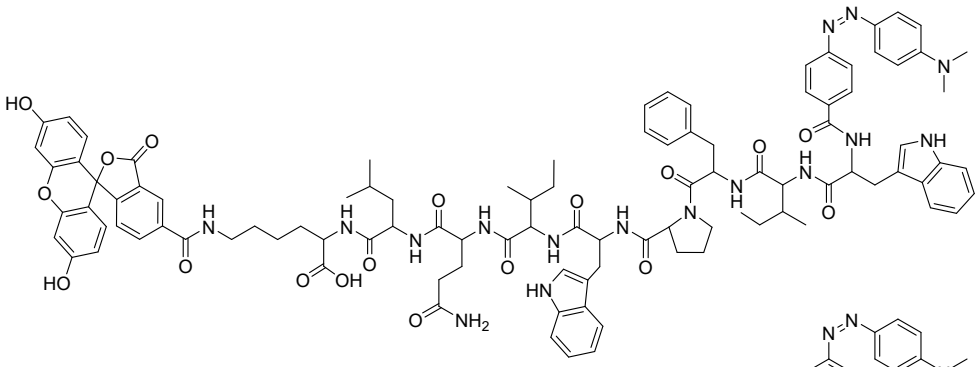
**Model mice and *in vivo* imaging.** All the animal experiments were approved by the Radiation Protection Institute of Drug Safety Evaluation Center in China (Production license: SYXK (Jin) 2018-0005). All the animal experiments were performed by following the protocols approved by the Radiation Protection Institute of Drug Safety Evaluation Center in China (Production license: SYXK (Jin) 2018-0005). The studies involving human participants were reviewed and approved by the Ethics Committee of the First Hospital of Shanxi Medical University, China (No. KYLL-2024-197). Female BALB/c nude mice of 6-8 weeks old were purchased from Beijing Viton River Experimental Technology Co., Ltd. The mice were housed under a 12-h light/dark cycle and were allowed free access to food and water. ACHN cells were injected into mice to obtain xenograft tumor bearing mice model. Mice were intratumor injection with 2 mM probes. Fluorescence images were acquired with time change via the following parameters: excitation wavelength: 465 nm; emission filter: 520 nm - 580 nm. Afterward, mice were euthanized by cervical dislocation, and organ were collected and imaged further.

**Tissue slice imaging of RCC**. All tissue slice samples from patients were harvested from First Hospital of Shanxi Medical University. The studies involving human participants were reviewed and approved by the Ethics Committee of the First Hospital of Shanxi Medical University, China (No. KYLL-2024-197). All patients gave their informed consent to participate in the research. All methods were performed in accordance with the relevant guidelines and regulations. Adjacent tissues were identified for longitudinal assessment of kidney function.

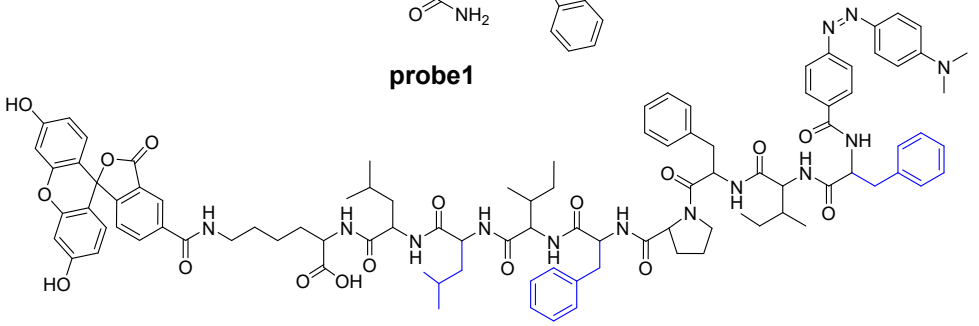
## 2. Synthesis and Characterization

**Synthesis details of probes.** The peptidyl probes (designated as **probe2-5**, as shown in Scheme S1), which introduced peptide sequence modified with 5-FAM fluorophore and dabcyl quencher, were custom made from Bankpeptide Biological

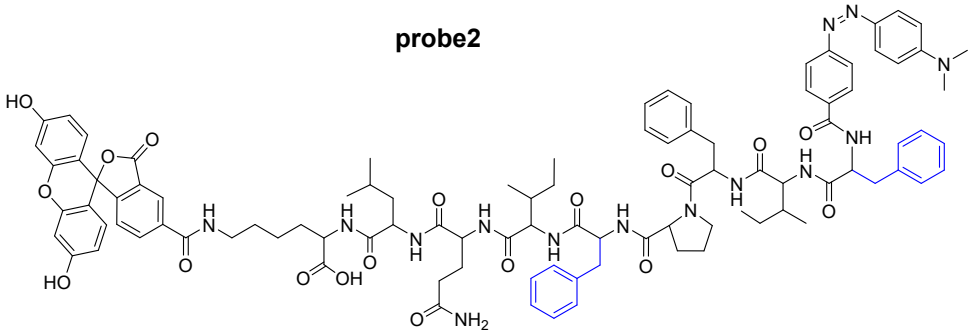
Technology Co., Ltd. **Probe1** was developed by Wen and colleagues to target GRP78, as described in their published work [S1].



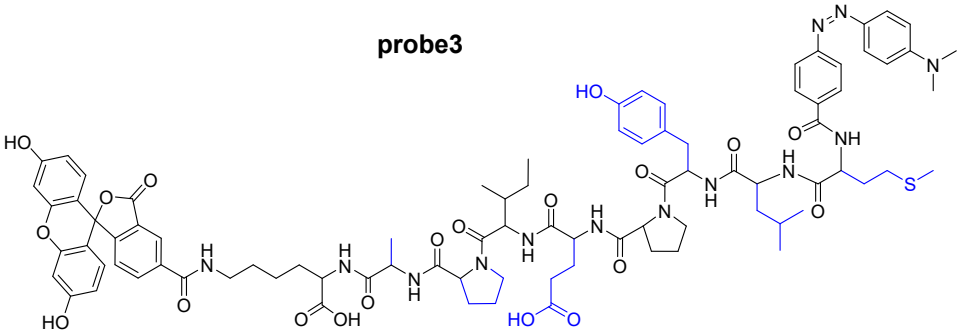
probe1



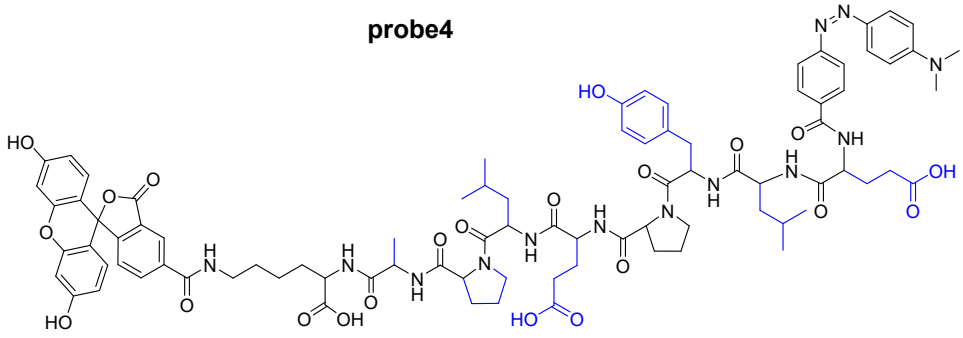
probe2



probe3



probe4



probe5

**Scheme S1.** Chemical structures of **probes1-5**.

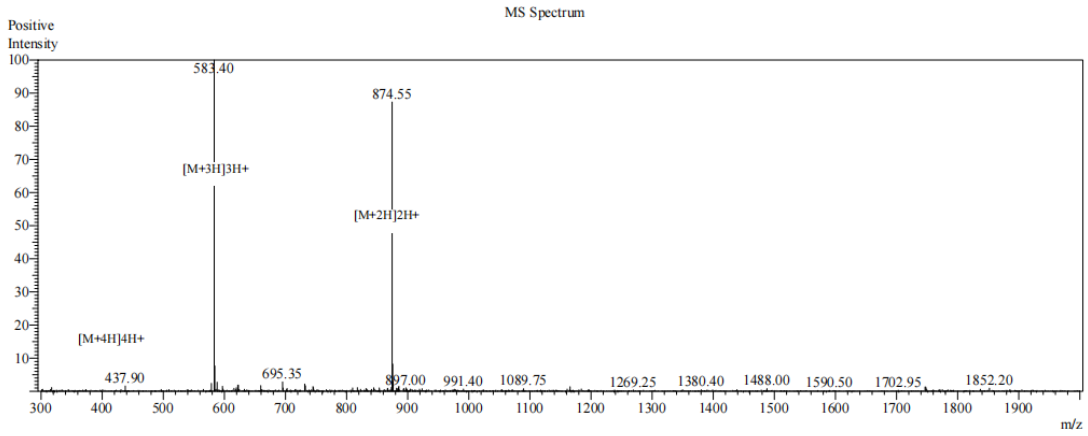
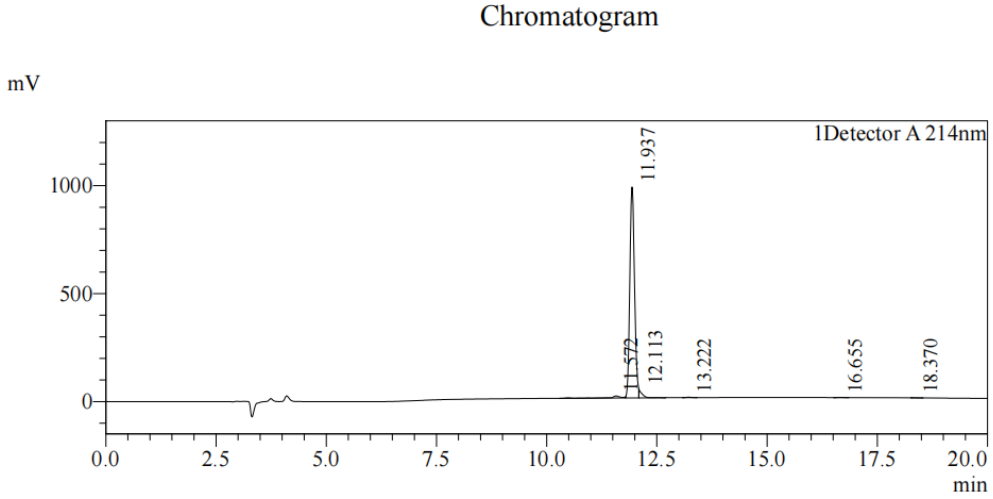



**Figure S1**. The HR-MS of **probe2**. Calc. for $C_{98}H_{118}N_{13}O_{17}^{2+}$ $[M+3H]^{2+}$ 874.44, found 874.55.




Peak Table

Detector A 214nm

| Peak# | Ret. Time | Area | Height | Area% |
|---|---|---|---|---|
| 1 | 11.572 | 148733 | 9540 | 1.940 |
| 2 | 11.937 | 7319228 | 976575 | 95.458 |
| 3 | 12.113 | 178458 | 32352 | 2.327 |
| 4 | 13.222 | 13235 | 1864 | 0.173 |
| 5 | 16.655 | 5164 | 679 | 0.067 |
| 6 | 18.370 | 2627 | 417 | 0.034 |
| Total | | 7667445 | 1021426 | 100.000 |

**Figure S2**. Typical chromatogram of **probe2**, which presented that the purification of the final probe >95%.

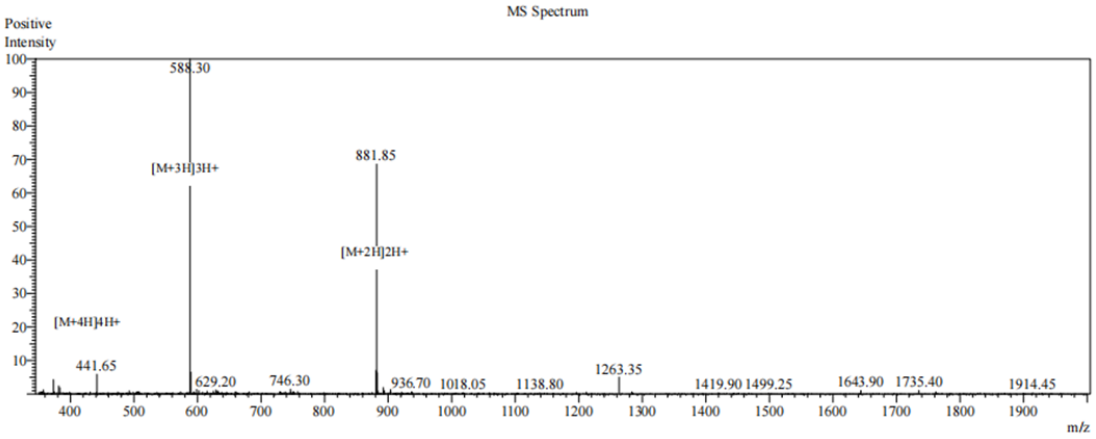
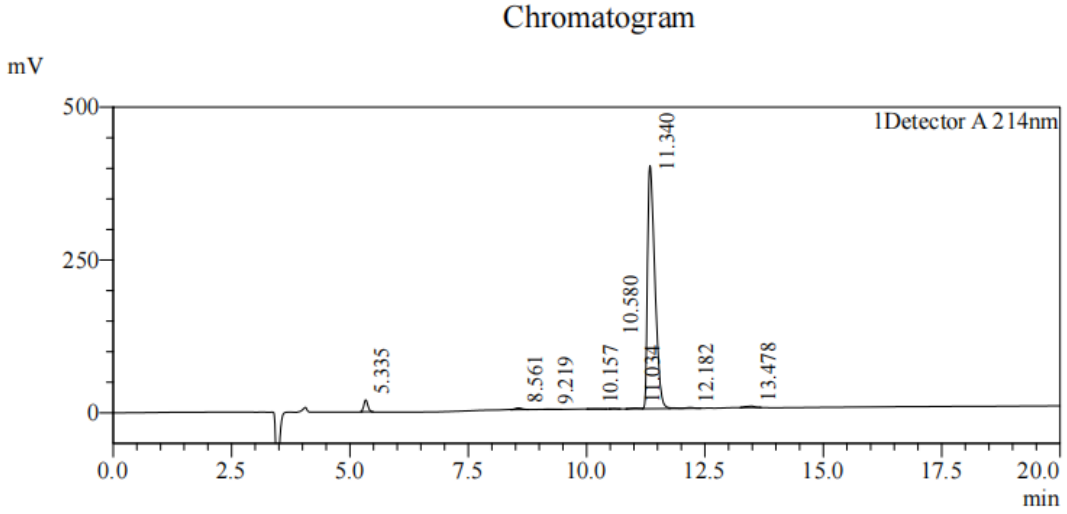



**Figure S3**. The HR-MS of **probe3**. Calc. for $C_{97}H_{115}N_{14}O_{18}^{2+}$ $[M+3H]^{2+}$ 881.93, found 881.85.




Peak Table

Detector A 214nm

| Peak# | Ret. Time | Area | Height | Area% |
|---|---|---|---|---|
| 1 | 5.335 | 112468 | 19308 | 2.492 |
| 2 | 8.561 | 24872 | 2661 | 0.551 |
| 3 | 9.219 | 4771 | 442 | 0.106 |
| 4 | 10.157 | 9614 | 814 | 0.213 |
| 5 | 10.580 | 3553 | 533 | 0.079 |
| 6 | 11.034 | 15817 | 1156 | 0.350 |
| 7 | 11.340 | 4291387 | 397512 | 95.072 |
| 8 | 12.182 | 22674 | 1167 | 0.502 |
| 9 | 13.478 | 28675 | 2232 | 0.635 |
| Total | | 4513830 | 425824 | 100.000 |

**Figure S4**. Typical chromatogram of **probe3**, which presented that the purification of the final probe >95%.

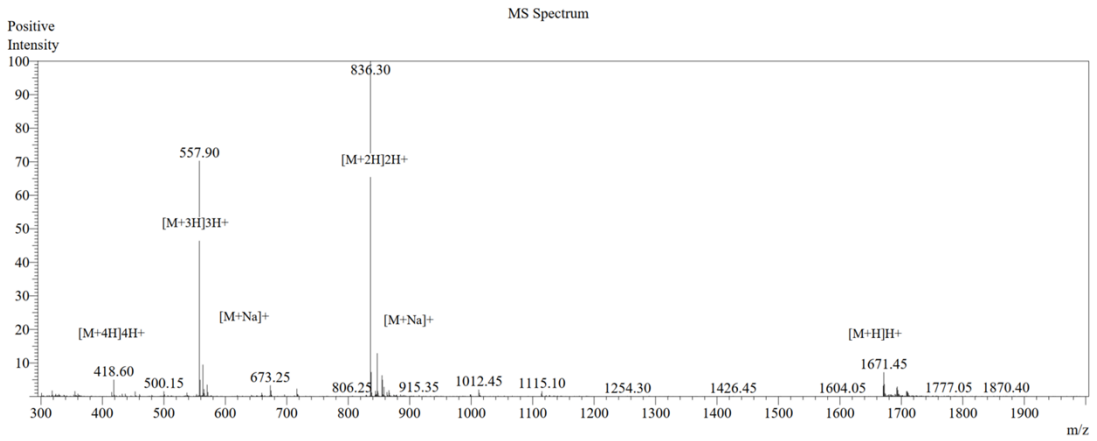
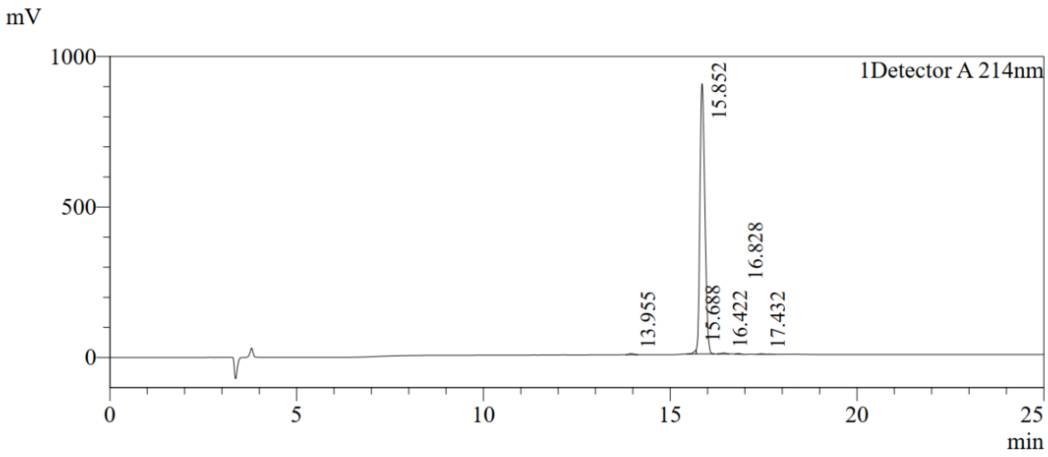



**Figure S5**. The HR-MS of **probe4**. Calc. for $C_{86}H_{106}N_{13}O_{20}S^{2+}$ $[M+3H]^{2+}$ 836.37, found 836.30.

Chromatogram




Peak Table

Detector A 214nm

| Peak# | Ret. Time | Area | Height | Area% |
|---|---|---|---|---|
| 1 | 13.955 | 29762 | 3766 | 0.357 |
| 2 | 15.688 | 63159 | 11500 | 0.757 |
| 3 | 15.852 | 8186517 | 898250 | 98.181 |
| 4 | 16.422 | 24310 | 2869 | 0.292 |
| 5 | 16.828 | 11752 | 1673 | 0.141 |
| 6 | 17.432 | 22723 | 1670 | 0.273 |
| Total | | 8338224 | 919728 | 100.000 |

**Figure S6**. Typical chromatogram of **probe4**, which presented that the purification of the final probe >95%.

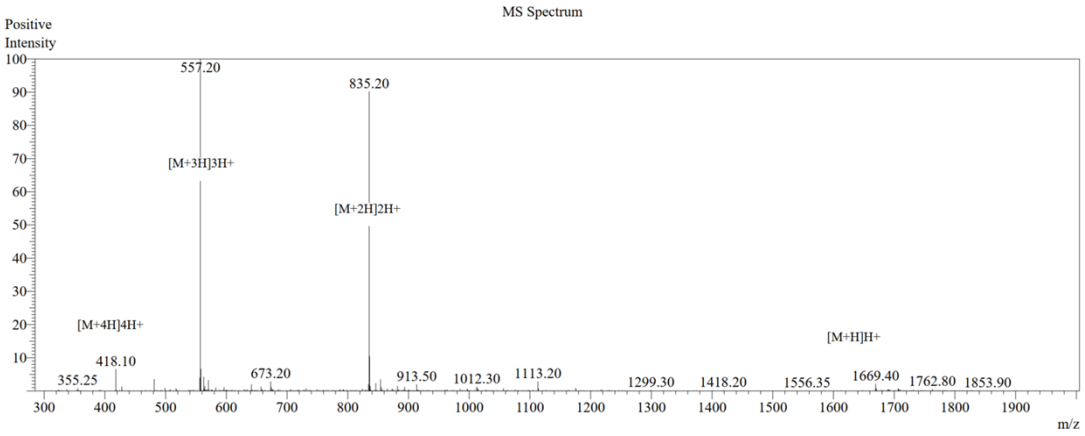
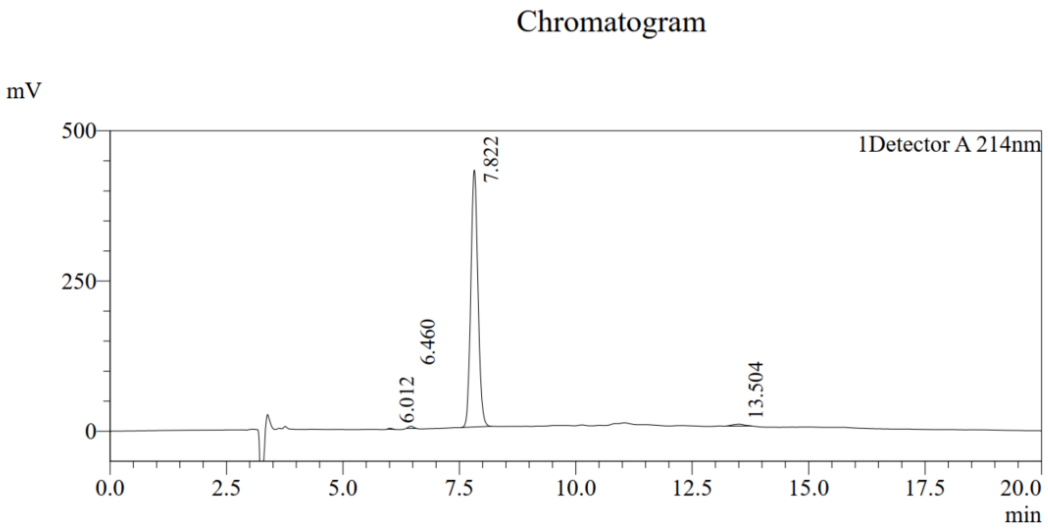



**Figure S7**. The HR-MS of **probe5**. Calc. for $C_{86}H_{104}N_{13}O_{22}^{2+}$ $[M+3H]^{2+}$ 835.37, found 835.20.




Peak Table

Detector A 214nm

| Peak# | Ret. Time | Area | Height | Area% |
|---|---|---|---|---|
| 1 | 6.012 | 8014 | 1497 | 0.173 |
| 2 | 6.460 | 25957 | 3565 | 0.559 |
| 3 | 7.822 | 4558341 | 427459 | 98.130 |
| 4 | 13.504 | 52840 | 2961 | 1.138 |
| 5 | 21.083 | 56 | 19 | 0.001 |
| Total | | 4645209 | 435502 | 100.000 |

**Figure S8**. Typical chromatogram of **probe5**, which presented that the purification of the final probe >95%.

## 3. Additional spectra data

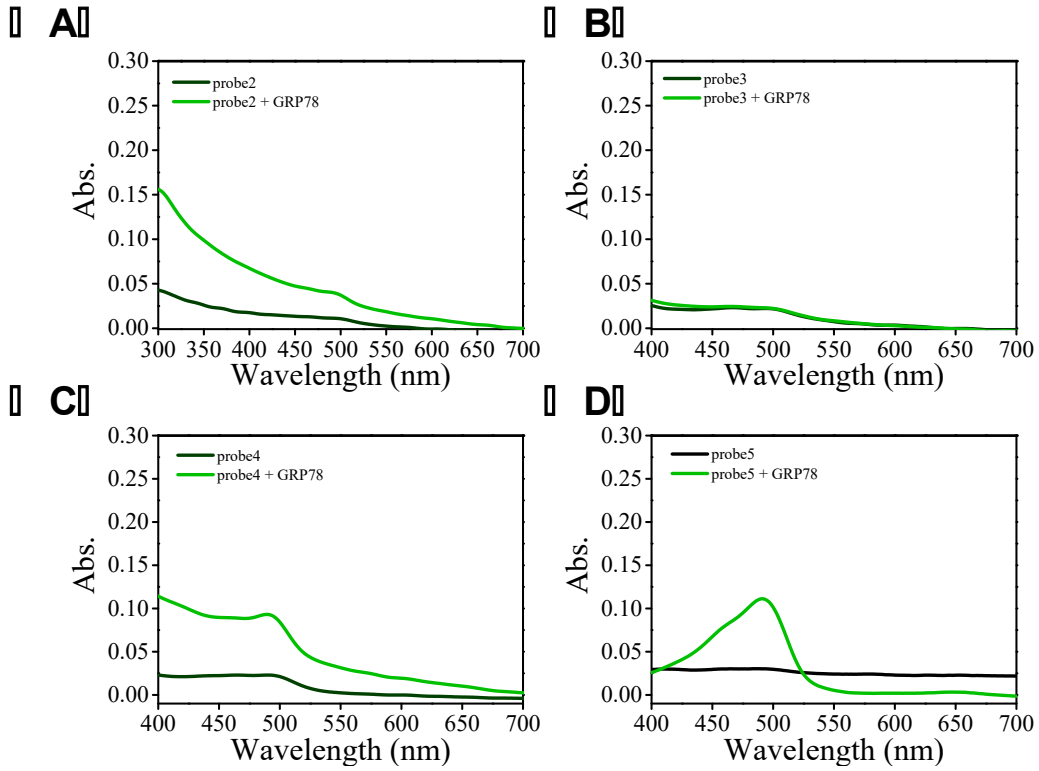



**Figure S9.** Absorption spectra of 5 μM **probe 2** (A), **probe 3** (B), **probe 4** (C) and **probe 5** (D) upon addition of 50 μg/mL GRP78 in 10 mM phosphate buffer (DMSO 0.25%, v/v, pH = 7.2).

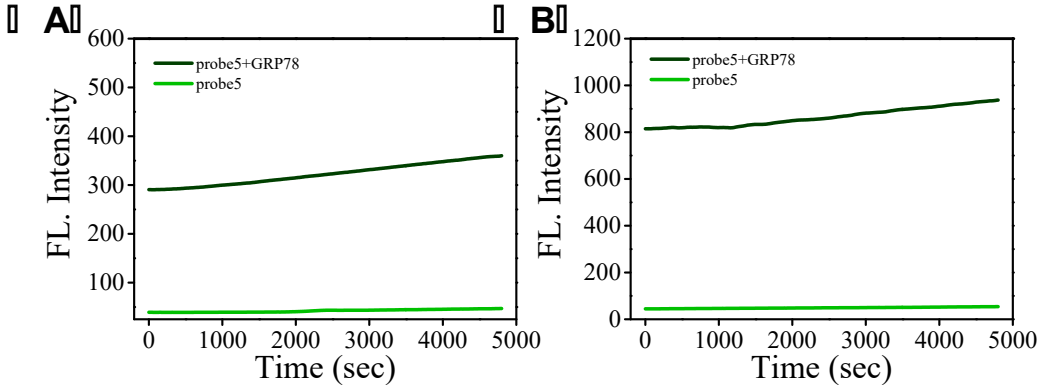



**Figure S10.** Change of fluorescence intensity at 528 nm of **probe 5** (5 μM) with/without 50 μg/mL GRP78 over time (A) in PBS buffer (10 % fetal bovine serum) and (B) in PBS buffer (10 % MEM medium), $\lambda_{ex}$= 490 nm.

## 4. Additional cells imaging data

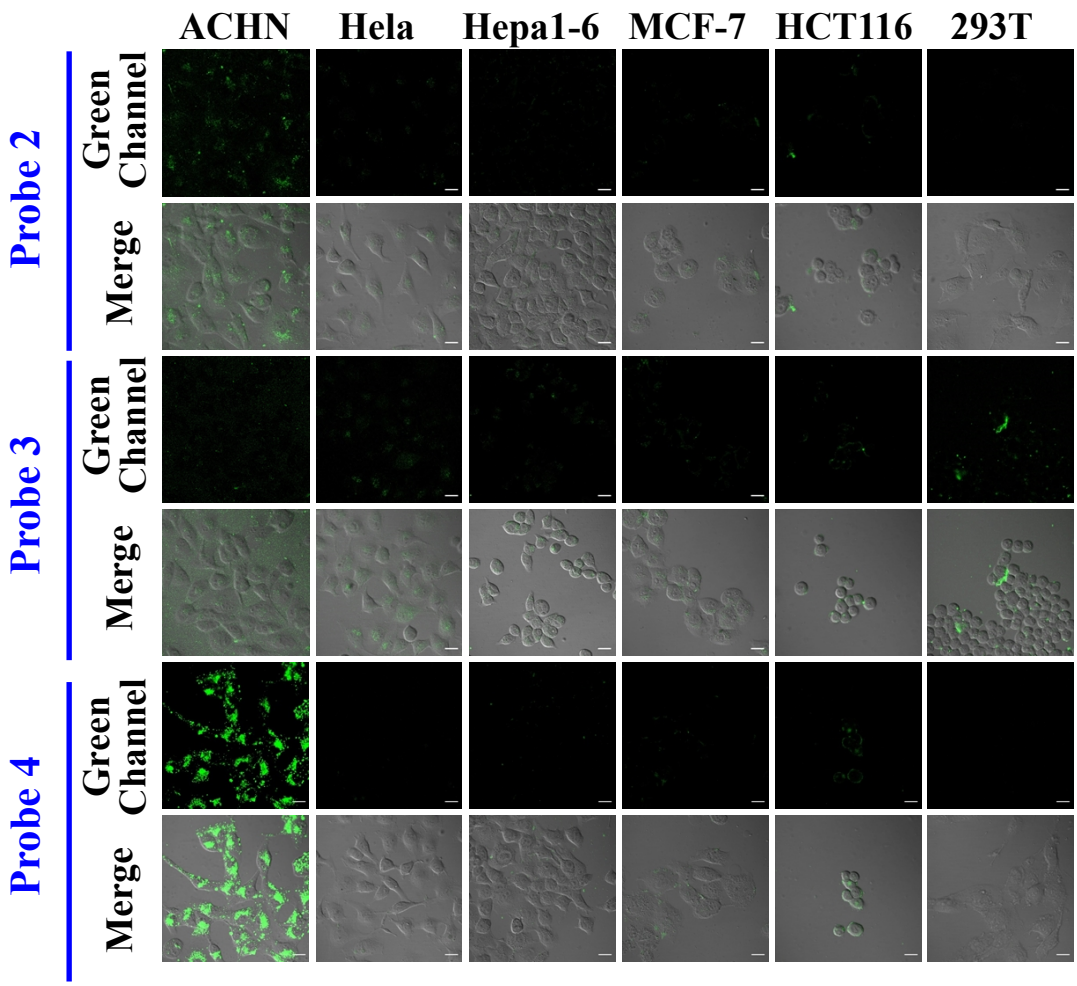



**Figure S11.** CLSM imaging with probes in ACHN, Hela, Hepa1-6, MCF-7, HCT116, 293T cells. Green channel ($\lambda_{ex}$ = 488 nm, $\lambda_{em}$ = 500-560 nm) imaging signal of cells preincubated with **probe2**, **probe3** or **probe4** (10 μM) were collected for 120 min. Scale bar = 10 μm.

## 5. Western blot assays

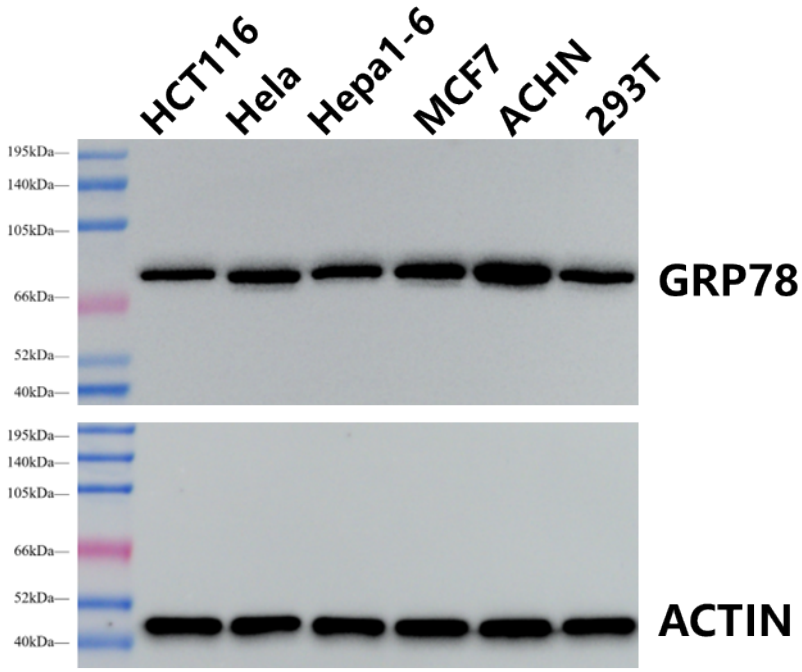



**Figure S12.** Expression of GRP78 in ACHN, HeLa, Hepa1-6, MCF-7, HCT116, and 293T cells.

## 6. Cell viability data

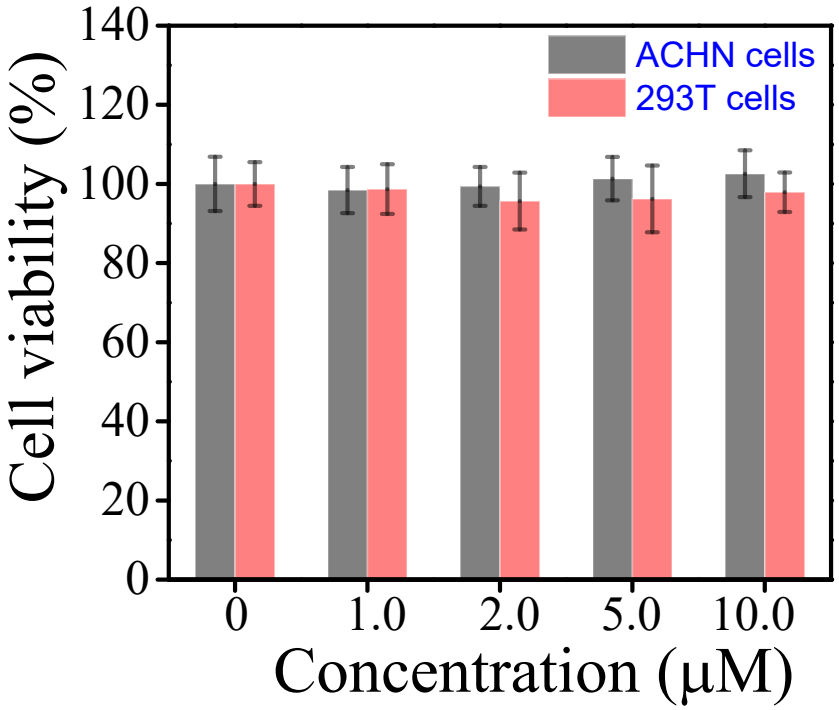



**Figure S13.** Cell viability values (%) estimated by MTT assay in ACHN cells and 293T cells, which were cultured in the presence of 0-10 μM **probe 5** for 5 h.

## 7. Additional *in vivo* imaging data

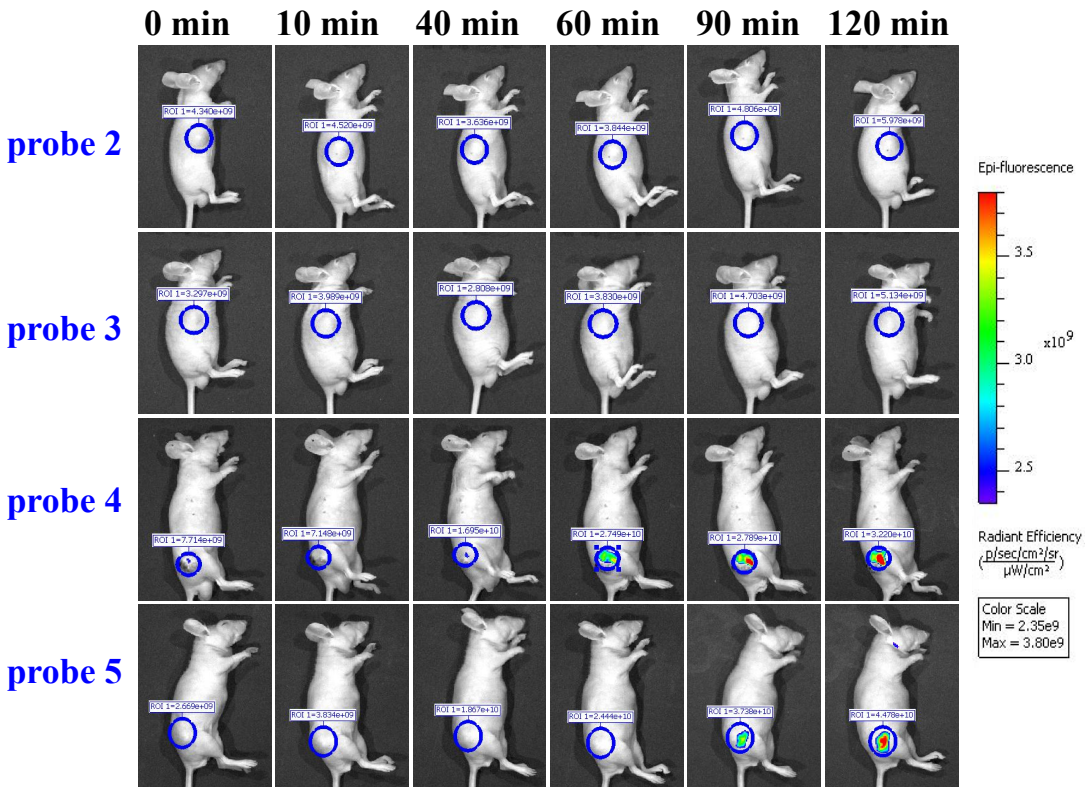



**Figure S14.** *In vivo* imaging of **probe2**, **probe3**, **probe4** and **probe5** (2 mM) in ACHN-bearing nude mice over time. Green channel: $\lambda_{ex}$ = 465 nm, $\lambda_{em}$ = 520–580 nm.

## 8. Additional References

S1 Y. Wen, N. Jing, F. H. Huo, C. X. Yin*, Rational design of a turn-on fluorescent probe for visualization of GRP78 protein in tumor models, *Chin. Chem. Lett.*, 2023, **34**, 107604.