Supporting Information

A Photostable Fluorescent Probe for Targetted Imaging of Tumour Cells Possessing Integrin $\alpha_v\beta_3$

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cyclo(Arg-Gly-Asp-Phe-Lys(mpa)) was kindly provided by GL Biochem (Shanghai) Ltd (Purity: 98.52%, Fig. S6 and Fig. S7). Acridine orange was purchased from JING TIAN BIO (Shanghai). All other chemical reagents were purchased from Sigma-Aldrich and were used as received. 8-Oxo-8H-acenaphtho[1,2-b]pyrrole-9-carbonitrile was synthesized according to the reference1.

**General Experiments:**

UV-visible absorption spectra were measured using a Shimadzu UV-2550 ultraviolet-visible-near infrared spectrophotometer. Fluorescence emission spectra were measured on an Edinburgh LFS920 fluorescence spectrometer with a 1000 W xenon lamp. Probe 2 was purified by reverse phase preparative HPLC: SunFire™ Prep C18, 4.6 mm × 150 mm, 5μm. The mobile phase was changed from 30% acetonitrile in water (0.05% TFA) to 40% acetonitrile in water (0.05% TFA) in 7 min, hold at 40% for 7 min. The flow rate was 40 mL/min and UV absorbance was monitored at 214 nm. Analytical purity was assessed by analytical reverse phase HPLC: XBRIDGE-C18, 4.6 mm × 100 mm, 3.5μm. The mobile phase was changed from 10% acetonitrile in water (0.05% TFA) (0–1.5 min) to 95% acetonitrile in water (0.05% TFA) at 16 min. The flow rate was 1.2 mL/min and UV absorbance was monitored at 214 nm.

**Synthesis of 2:**

To a solution of 8-Oxo-8H-acenaphtho[1, 2-b]pyrrole-9-carbonitrile (0.0115 g, 50 μmol) in DMF (3 mL), c(RGDFK)-SH (0.0692 g, 100 μmol) was added, and then the mixture was stirred at room temperature. After a few minutes, the solution became red and was left to react for 2 hours. The product was purified by reverse phase preparative HPLC. The fractions containing pure 2 were collected, freeze-dried and then stored at −20 °C (0.025 g, 55%). MS (m/z) calcd. for C45H49N11O9S [M+H]+ 920.34; found 920.3.

**Cell Culture:**

The HeLa (human cervical carcinoma) and SMMC-7721 (human hepatocellular carcinoma) cell lines were provided by Institute of Basic Medical Sciences Chinese Academy of Medical
Sciences. The HeLa cells were grown in MEM (Modified Eagle’s Medium) supplemented with 10% FBS (Fetal Bovine Serum), and the SMMC-7721 cells were grown in RPMI 1640 supplemented with 10% FBS. Cultures were maintained at 37°C under a humidified atmosphere containing 5% CO₂.

**Cytotoxicity Assay:**

The in vitro cytotoxicity was measured using the methyl thiazolyl tetrazolium (MTT) assay in human hepatocellular carcinoma cell line SMMC-7721. Cells growing in log phase were seeded into 96-well cell-culture plate at $1 \times 10^4$/well and then incubated for 24 h at 37 °C under 5% CO₂. The compound 1 (100 µL/well) at concentrations of 5, 25, 50, 100 µM were added to the wells of the treatment group, and 100 µL/well DMSO diluted in RPMI 1640 at final concentration of 0.2% to the negative control group, respectively. The cells were incubated for 24 h at 37 °C under 5% CO₂. Subsequently, 10µL MTT (5 mg/mL) was added to each well of the 96 well assay plate and incubated for an additional 4 h at 37 °C under 5% CO₂. After the addition of 10% Sodium dodecyl sulfate (SDS, 100 µL/well), the assay plate was allowed to stand at room temperature for 12 h. Tecan Infinite M200 monochromator-based multi-function microplate reader was used to measure the OD570 (A value) of each well with background subtraction at 690 nm. The following formula was used to calculate the viability of cell growth:

$$\text{cell viability (\%) = (mean of Absorbance value of treatment group} / \text{mean of Absorbance value of control)} \times 100.$$  

**Fluorescence Imaging:**

Confocal fluorescence imaging, including xy-scan and spectrum-scan were performed with an OLYMPUS FV1000 laser scanning microscope and a 60× oil-immersion objective lens (1.35 numerical aperture, OLYMPUS, Japan).

For cell imaging, cells ($5 \times 10^5$/L) grown on 18 mm glass coverslips were washed with PBS and then were incubated with 20 µM 2 under serum-free medium at 37°C for 30 min. For competition studies, the cells were preincubated with 200 µM unlabeled c(RGDFK) for 30 min before the addition of 20 µM 2. The cells were further incubated at 37°C for 30 min. For
4°C experiment, after washing with PBS, cells were incubated in serum-free medium at 4°C for 10 min and then were incubated with 20 µM 2 under serum-free medium at 4°C for 30 min. Cell imaging was then carried out after washing cells with PBS. Cells loaded with 2 were excited at 515 nm using a multiline argon ion laser. Emission was collected from 535 to 635 nm.

For dual-labeled imaging in the photobleaching experiments under the same excitation conditions, acridine orange and 2 were excited using a multiline argon ion laser at 488 nm. Emission of acridine orange was collected at 500-540 nm in channel-1, emission of 2 was collected at 560-600 nm in channel-2. After washing with PBS, cells were incubated with 20 µM 2 under serum-free medium at 37°C for 1h. Subsequently, the cells were washed with PBS and then incubated with 0.5 µg/mL acridine orange in a serum-free medium for 1h at 37°C. Cell imaging was then carried out after washing cells with PBS. Quantization by line plots and fluorescence decay were analyzed using software package provided by OLYMPUS instrument.

**Flow Cytometry Assay:**

SMMC-7721 cells and HeLa cells were detached by pipetting and by harvesting with trypsin, respectively. Both types of cells were washed twice with PBS. Cells pellets (1 × 10^6 cells) were incubated with compound 2 (20 µM) for 30 min and 2 h at 37°C. After washing, the cells were resuspended in PBS and analyzed with a flow cytometry FACSCalibur. The collected data were analyzed using CELLQuest software (Becton–Dickinson, CA, USA).
Fig. S1. The HPLC-MS chromatogram of 2.
Fig. S2. Fluorescence spectra obtained from the living SMMC-7721 and HeLa cells incubated with 2 (20 µM) for 1 h at 37 °C (λ<sub>ex</sub> = 515 nm).

Fig. S3. Confocal fluorescence (a and b) and brightfield (c) images of the SMMC-7721 cells stained with 20 µM 2 and 0.5 µg/mL acridine orange (λ<sub>ex</sub> = 488 nm). The signals of acridine orange and 2 were collected from the green channel (channel-1: 520 ± 20 nm) and red channel (channel-2: 580 ± 20 nm), respectively. Overlay of panels (a), (b) and (c) is shown in panel (d).
Fig. S4. The change in the fluorescence spectra of 2 (20 μM) in DMSO/PBS (1:119, v/v) (λ<sub>ex</sub> = 515 nm).

Fig. S5. The structure of acridine orange.
Fig. S6. The HPLC chromatogram of c(RGDFK)-SH.

Results

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Sample Description:
- Structure: c(RGDFK)-SH
- Lot No.: P071114-SY105524
- Column: 4.6×250mm, GraceSmart RP18-5
- Solvent A: 0.1% trifluoroacetic in 100% acetonitrile
- Solvent B: 0.1% trifluoroacetic in 100% water
- Gradient: 0.01min 15% 85% 25min 40% 60% 35.5min 100% 0%
- Flow rate: 1.0 mL/min
- Wavelength: 220nm
- Volume: 10μl
Fig. S7. The MS spectrum of c(RGDFK)-SH.

[Image of MS spectrum with peaks labeled and a table of sample information]