Supporting information

A Nile Blue Based Infrared Fluorescent Probe: Imaging tumors that over-express Cyclooxygenase-2

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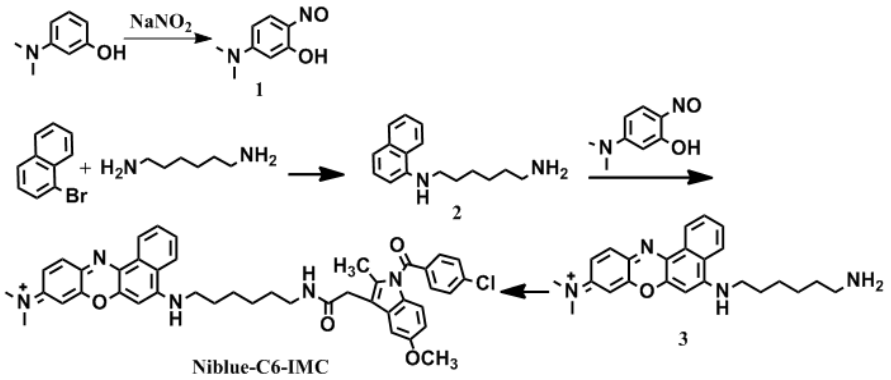
Content

Materials and methods

All solvents and reagents used were reagent grade, and were used without further purification. The solution of **Niblue-C6-IMC** was dissolved in dimethyl sulphoxide (DMSO) at a concentration of 1mM as the stock solution. ^{1}H NMR and ^{13}C NMR spectra were recorded on a VARIAN INOVA-400(or a Bruker Avance II 400 MHz) spectrometer. Chemical shifts (δ) were reported as ppm (in $CDCl_3$ or DMSO, with TMS as the internal standard). Mass spectrometric data were achieved with HP1100LC/MSD MS and an LC/Q-TOF-MS instruments. Fluorescence spectra were performed on a VAEIAN CARY Eclipse fluorescence spectrophotometer (Serial No. FL0812-M018). Excitation and emission slit widths were modified to adjust the fluorescence intensity to a suitable range. Absorption spectra were measured on a Perkin Elmer Lambda 35 UV/VIS spectrophotometer. NBD C6-ceramide was purchased from Life Technologies Co. (USA). Tris base was from Promega Co. (USA). Cyclooxygenase-2 (COX-2) was obtained from Sigma Chemical Co. (USA). Mice with tumors (MDA-MB-231 and S180 sarcoma) were purchased from Slac Laboratory Animal Co. (Shanghai, China).

Synthesis of Compounds



Scheme S1. Synthetic procedures of **Niblue-C6-IMC** and its intermediates.

The synthesis of 1

1 was synthesized from 3-(dimethylamino) phenol by the procedure published in literature[1].

The synthesis of 2

To a 100 ml flask, 1-bromonaphthalene (4.12 g, 19.90 mmol) and 1,6-diaminohexane (4.65 g, 40.02 mmol) was dissolved in 40 mL 2-methoxyethanol, then CuI (190 mg, 1.0 mmol) and $CsCO_3$(3.0 g, 15.55 mmol) was added and the mixture was refluxed at 125℃ for 24 h. After completion of the reaction by TLC, the solid was precipitated and filtered off to yield pale yellow solution. The crude product was purified by silica gel column chromatography with CH_2Cl_2/CH_3OH (20/1) and gets the desired yellow oil product (2.5 g, 52%). ^{1}H NMR (400 MHz, CD_3OD), δ 8.00 (m, 1H), 7.70 (m, 1H), 7.38 (m, 2H), 7.27 (t, J = 8 Hz, 1H), 7.13 (d, J = 8 Hz, 1H), 6.57 (d, J = 8 Hz, 1H), 3.23 (t, J = 8 Hz, 2H), 2.60 (t, J = 8 Hz, 2H), 1.74-1.36 (m, 8H). ^{13}C NMR (100 MHz, CD_3OD) δ: 144.0, 134.5, 127.9, 126.3, 125.1, 123.9, 123.7, 120.5, 116.1, 103.6, 43.6, 40.8, 31.6, 28.6, 26.9, 26.4 ppm. TOF MS: m/z calcd for $C_{16}H_{22}N_2$ $[M+1]^+$: 243.25, found: 242.18.

The synthesis of 3

To a cold solution (ice bath) of 2 (770.0 mg, 3.18 mmol) in ethanol (20.0 mL), compound 1 (528.4 mg, 3.18 mmol) and concentrated hydrochloride acid (1.0 mL) were added. The mixture was stirred for 10 minutes, then refluxed at 90 ℃ for 2.5 h and monitored by TLC (CH_2Cl_2:CH_3OH=10:1). The compound 3 was obtained by recrystallization with ethanol. Then, the suspension was filtrated and purified by silica gel column chromatography with CH_2Cl_2/CH_3OH (6/1) and get the desired violet solid product (441 mg, 49%). ^{1}H NMR (400 MHz, CD_3OD), δ: 8.94 (d, J = 8 Hz,1H), 8.42 (d, J = 8 Hz, 1H), 7.96 (m, 1H), 7.88 (m, 2H), 7.29 (m, 1H), 7.04(s, 1H), 6.92(d, J =2.8 Hz, 1H), 3.79 (t, J = 8 Hz, 2H), 3.33 (s, 6H), 2.96 (t, J =8 Hz, 2H), 1.93-1.56 (m, 8H). ^{13}C NMR (100 MHz, CD_3OD), δ: 25.8, 26.2, 27.1, 28.1, 39.65, 95.81, 96.38, 108.11, 114.88, 124.40, 124.41, 129.63, 132.32, 132.47, 133.15, 133.80, 148.55, 151.92, 156.80, 157.58 ppm; TOF MS: m/z calcd for $C_{24}H_{29}N_4O^+$ $[M]^+$: 389.2323, found: 389.2336.

The synthesis of Niblue-C6-IMC

To a solution of 3 (120 mg, 0.308 mmol) in 10ml DMF, indomethacin (110.23 mg, 0.308 mmol), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCl) (70 mg, 0.370 mmol), HOBt·H_2O (70 mg, 0.462 mmol) and 4-dimethylaminopyridine (DMAP) (45 mg, 0.370 mmol) were added. The solution was stirred at room temperature under nitrogen for 24 h and then concentrated. The residue was purified by silica gel column chromatography with CH_2Cl_2/CH_3OH (20:1), affording **Niblue-C6-IMC** as violet solid (148 mg, 66%). ^{1}H NMR (400 MHz, CD_3OD)，δ: 8.75 (d, J = 8 Hz , 1H), 8.22 (d, J = 8 Hz , 1H), 7.86 (t, J = 8 Hz , 2H), 7.73 (q, 3H), 7.45 (m, 4H), 7.14 (d, J = 8 Hz, 1H), 6.69 (d, J = 4 Hz, 1H), 6.66 (s, 1H), 6.58 (d, J = 8 Hz, 1H), 6.37 (d, J = 4 Hz, 1H), 3.64 (s, 3H), 3.56 (s, 2H), 3.51 (t, J = 8 Hz, 2H), 3.25 (s, 6H), 2.22 (s, 3H), 1.75–1.40(m, 10H); ^{13}C NMR (100 MHz, CD_3OD), δ: 171.8, 168.1, 156.0, 155.7, 151.7, 147.7, 138.7, 135.5, 134.0, 132.2, 131.6, 131.1, 130.8, 130.0, 129.6, 128.7, 126.6, 125.6, 124.3, 123.4, 122.5, 117.2, 115.0, 114.3, 113.8, 110.8, 110.1, 101.2, 95.8, 93.0, 54.6, 39.7, 38.6, 31.0, 28.9, 27.9, 26.7, 25.8, 25.5, 12.3 ppm; TOF MS: m/z calcd for $C_{43}H_{43}ClN_5O_4^+$ $[M]^+$: 728.2998, found: 728.2993.

Photophysical properties of Niblue-C6-IMC

Absorption UV-visible spectra spectra were collected on a HP-8453 spectrophotometer (Agilent, USA). Fluorescence spectra were obtained with a FP-6500 spectrophotometer (Jasco, Japan) with slit widths were set at 2.5 nm and 5 nm for excitation and emission, respectively. The relative fluorescence quantum yields of **Niblue-C6-IMC** in different kinds of solution were determined according to the method below[2].

$$\varphi_u = \frac{(\varphi_s)(FA_u)(A_s)(\lambda_{exs})(\eta_u^2)}{(FA_s)(A_u)(\lambda_{exu})(\eta_s^2)}$$

Where φ is fluorescence quantum yield; *F*A is integrated area under the corrected emission spectra; A is the absorbance at the excitation wavelength; λ_{ex} is the excitation wavelength; η is the refractive index of the solution; the subscripts u and s refer to the unknown and the standard,

respectively. We chose rhodamine B as standard, which has the fluorescence quantum yield of 0.49 in ethanol[3].

Cell incubation and staining with Niblue-C6-IMC

Human cervical cancer cells (Hela), Human breast cancer cells (MCF-7), Human hepatoma cells (HepG2), Human normal liver cells (LO-2), Human osteoblasts (OB) and African green monkey kidney cells (COS-7) were purchased from Institute of Basic Medical Sciences (IBMS) of the Chinese Academy of Medical Sciences. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10 % fetal bovine serum (Invitrogen). The cells were seeded in 24-well flat-bottomed plates and then incubated for 24 h at 37 ºC under 5% CO_2. Before imaging, the live cells were incubated with 2.5 μM **Niblue-C6-IMC** for another 30 min and then washed with phosphate-buffered saline (PBS) three times. Fluorescence imaging was performed using an OLYMPUSFV-1000 inverted fluorescence microscope with a 60×objective lens.

Native-PAGE

MCF-7 cells were incubated at 37 ºC under 5% CO_2 with **Niblue-C6-IMC** at varying concentrations (0, 1.0, 2.5, 5.0, 10.0 μM) for 30 min. For competitive experiments, cells were preincubated with 13.0 μM celecoxib for 3 h，and then added 5 μM **Niblue-C6-IMC** for another 30 min. When digested by pancreatin, the cells fell off from culture flask, and then treated with 1mL lysate and tissue proteomes. After concentrate through the overspeed centrifugal, the protein extracts were obtained. Add 25 μL 5 × Native-PAGE buffers (0.2 M Tris / 40 % glycerol / 0.4 % bromophenol blue) into 100 μL protein supernatant for gel electrophoresis. Bands were visualized by staining with Coomassie blue staining. For fluorescent labeling, the protein bands were visualized using a NightOWL II LB983 small animal in vivo imaging system containing a sensitive Charge Coupled Device (CCD) camera, with an excitation laser of 630 nm and an emission filter of 680 nm.

Fluorescence image of live cells stained with NBD C6-ceramide

Niblue-C6-IMC (2.5 μM) and NBD C6-ceramide (5.0 μM) were added to cancer cells. Cells were incubated for 30 min at 37 ºC under 5 % CO_2 and then washed with phosphate-buffered saline (PBS) three times. Fluorescence imaging was then carried out with OLYMPUSFV-1000 inverted fluorescence microscope, using a 60×objective lens. **Niblue-C6-IMC** (red channel) was excited at 635 nm, and the emission spectra were collected at 640-700 nm. NBD C6-ceramide (green channel) was excited at 488 nm, and the emission spectra were collected at 500-540 nm.

Cytotoxicity experiments

Measurement of cell viability was evaluated by reducing of MTT (3-(4, 5)-dimethylthiahiazo (-2-yl)-3, 5-diphenytetrazoliumromide) to formazan crystals using mitochondrial dehydrogenases

(Mosmann, 1983). MCF-7 cells were seeded in 96-well microplates (Nunc, Denmark) at a density of 1×10^5 cells/mL in 100 µL medium containing 10 % FBS. After 24 h of cell attachment, the plates were then washed with 100 µL / well PBS. The cells were then cultured in medium with 2.5 µM of **Niblue-C6-IMC** for 24 h. Cells in culture medium without **Niblue-C6-IMC** were used as the control. Six replicate wells were used for each control and test concentration. 10 µL of MTT (5 mg/mL) prepared in PBS was added to each well and the plates were incubated at 37ºC for another 4 h in a 5% CO_2 humidified incubator. The medium was then carefully removed, and the purple crystals were lysed in 200 µL DMSO. Optical density was determined on a microplate reader (Thermo Fisher Scientific) at 570 nm with subtraction of the absorbance of the cell-free blank volume at 630 nm. Cell viability was expressed as a percent of the control culture value, and it was calculated using the following equation:

Cells viability (%) = $(OD_{dye} - OD_{K\ dye})/ (OD_{control} \cdot OD_{K\ control}) \times 100$

Flow cytometry

MCF-7 cells were cultured in S6 RPMI 1640 supplemented with 10% FBS (fetal bovine serum) under an atmosphere of 5 % CO_2 and 95 % air at 37 °C. For flow cytometry studies, macrophages in the exponential phase of growth were plated into 35-mm glass-bottom culture dishes (Φ20 mm) containing 2.0 mL of RPMI 1640. After incubation at 37 °C with 5 % CO_2 for 1-2 days to reach 70-90 % confluency, the medium was removed. Then the cells were washed with 2.0 mL of PBS buffer, and 2.0 mL of fresh RPMI 1640 was added along with Lyso-NINO and/or iNOS stimulants. 1×10^5 cells were plated into a six-chamber culture well and incubated for 24 h. **Niblue-C6-IMC** was added to the culture medium, and the cells were incubated for 30 min. A 633 nm argon ion laser was used for excitation. Signals from cells were collected at 670 nm. Cells were analyzed in a FAC Scan cytometer (Becton Dickinson Biosciences Pharmingen, USA), and all data were analyzed with Cell Quest software.

ELISA to determine the amount of COX-2

This assay used the ABC-double antibody sandwich Enzyme linked immunosorbent assay (ELISA) method. COX-2 kit (human, double antibody method, 96 t) was purchased from CBS. Cells were prepared as homogenates, and preserved at 2-8ºC. The ELISA assay experiment was carried out according to literature procedures [4-5].

Preparation of tissue slices and staining with Niblue-C6-IMC

Tumour tissue slices were prepared from nude mice with S180 sarcoma. Normal tissue slices were prepared from livers of nude mice. The slices were cut at 800 µm using a vibrating-blade microtome in artificial cerebrospinal fluid (124 mM NaCl, 3 mM KCl, 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 10 mM D-glucose, 2.4 mM $CaCl_2$, and 1.3 mM $MgSO_4$). Then incubated tissue slices with **Niblue-C6-IMC** (100 µM) in PBS bubbled with 95 % O_2 and 5 % CO_2 for 30 min at 37 °C. After transferred to a glass bottomed dish (MatTek, 35 mm dish with 20 mm well), the tissue

slices were imaged using OLYMPUSFV-1000 inverted fluorescence microscope with a 635 nm excitation laser and emission spectra were collected at 640-700 nm.

Fluorescence imaging in mice

All procedures were carried out in compliance with the Guide for the Care and Use of Laboratory Animal Resources and the National Research Council, and were approved by the Institutional Animal Care and Use Committee of the NIH. Human breast cancer cell lines MDA-MB-231 were used for in vivo studies. The tumour implants were established by subskin injection of 1×10^6 to 2×10^6 cells suspended in 200 to 300 μL of PBS in BALB/c nude mice. Experiments with tumour-bearing mice were performed about 10 days, when implants grew up to about 0.5 cm in size. The mice with tumours were given a subcutaneous injection of **Niblue-C6-IMC** (100 μM) at about 2 cm away from the tumour region, near the enterocoelia. After injected **Niblue-C6-IMC** 60 min, the mice were imaged using a NightOWL II LB983 small animal in vivo imaging system with a 630 nm excitation laser and a 700 nm emission filter.

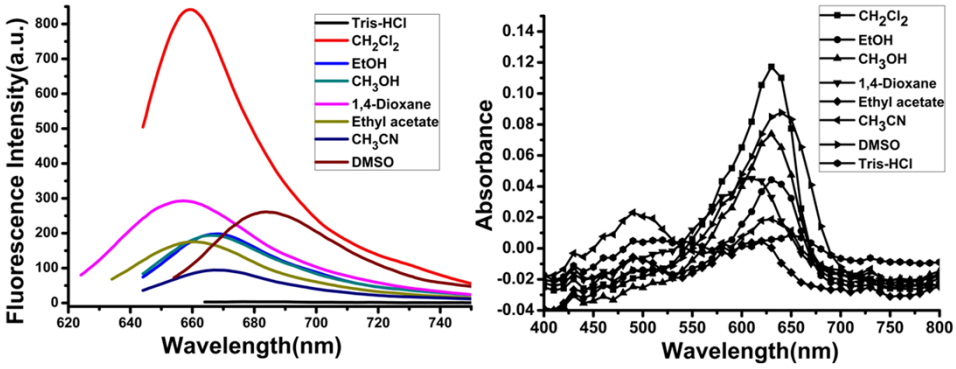


Fig. S1 The absorbance and emission spectra of **Niblue-C6-IMC** (5 μM) in different solutions.

Table S1 Photophysical properties of **Niblue-C6-IMC** in various solvents

Solvent	$\lambda_{abs(nm)}$[a]	$\lambda_{em(nm)}$[b]	$\Delta\lambda_{(nm)}$	ε(×10^5 L/mol cm)[c]	Φ[d]
CH_2Cl_2	629	660	31	0.530	0.52
EtOH	632	669	37	0.413	0.21
CH_3OH	631	667	36	0.570	0.15
1,4-Dioxane	483	613	130	0.281	0.03
Ethyl acetate	614	663	49	0.308	0.42
CH_3CN	629	668	39	0.375	0.23
DMSO	641	682	41	0.254	0.26
Tris-HCl[e]	644	684	40	0.130	0.05

[a, b] λ_{max} of the absorption and emission spectra in nm. [c] Fluorescence quantum yield. [d] Molar extinction coefficient. [e] Tris-HCl buffer (100 mM, pH = 8.0).

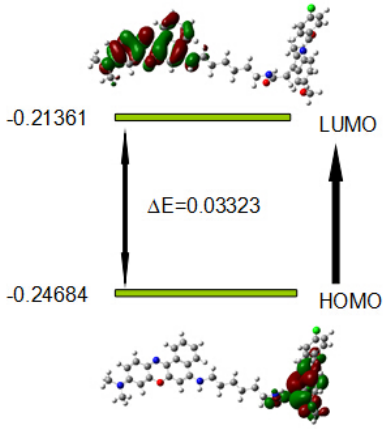


Fig. S2 Frontier molecular orbital (MO) of **Niblue-C6-IMC** calculated with time-dependent density functional theory using Gaussian 09.

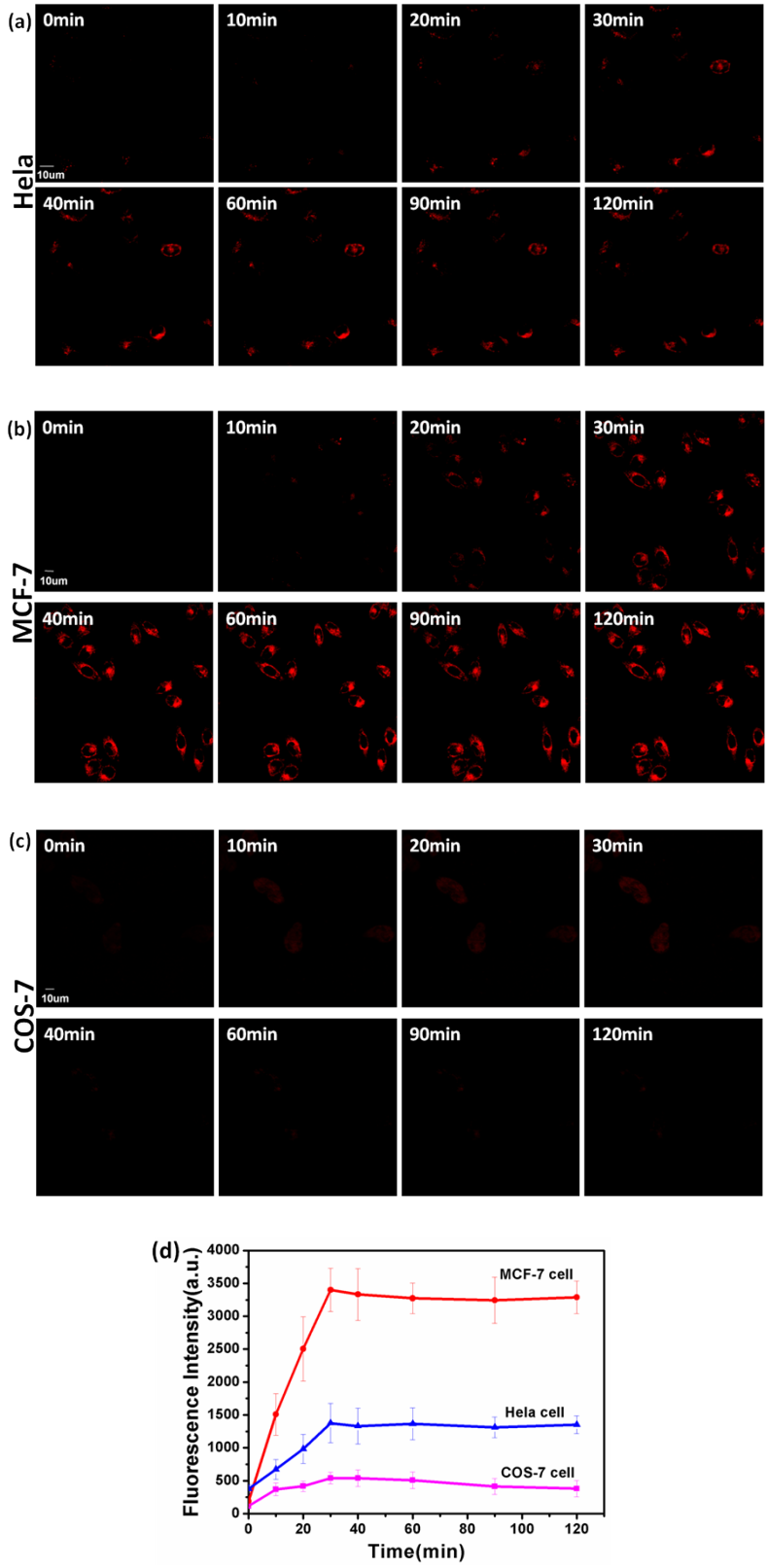


Fig. S3 Real-time fluorescence images of **Niblue-C6-IMC** in live a) Hela; b) MCF-7; c) COS-7 cells. Cells were cultured with 2.5 μM **Niblue-C6-IMC** and then confocal fluorescence images were recorded at different time points. Images were acquired by using excitation and emission windows of λ_{ex} = 635 nm and λ_{em} = 640–700 nm, respectively; d) Quantitative image analysis of the average total fluorescence of cells, determined from analysis of 10 cells in each sample image.

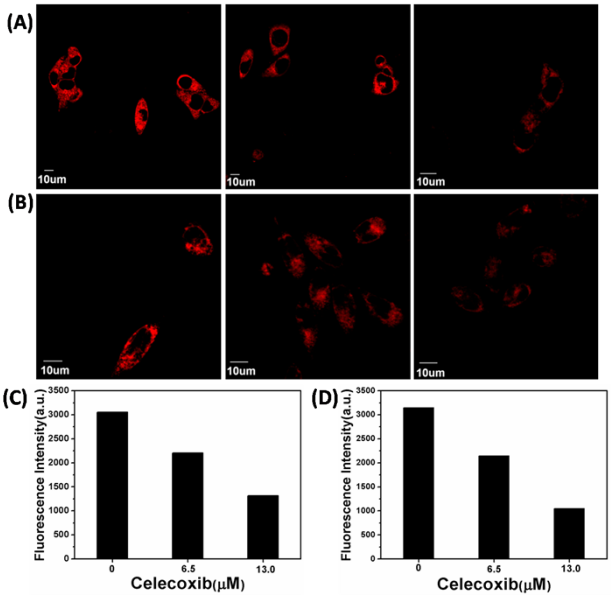


Fig. S4 Labeling of COX-2-expressing cells by **Niblue-C6-IMC**. Hela cells (A) and MCF-7 cells (B) pretreated with 0, 6.5 or 13.0 μM celecoxib for 3 h prior to **Niblue-C6-IMC** treatment. Excitation wavelength = 635 nm, scan range = 640-700 nm; Quantitative image analysis of the average total fluorescence of Hela cell (C) and MCF-7 cell (D), determined from analysis of 10 cells in each sample image.

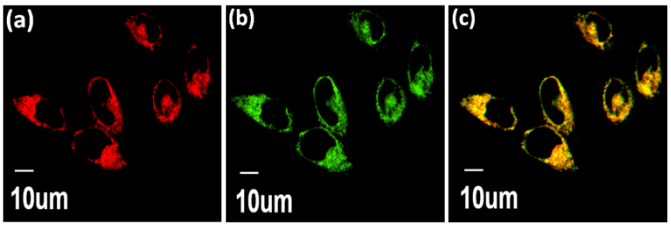


Fig. S5 Fluorescence images of **Niblue-C6-IMC** (2.5 μM) in HepG2 cells co-stained with NBD C6-ceramide (5.0 μM). (a) Red emission from **Niblue-C6-IMC**, excitation wavelength = 635 nm, scan range = 640-700 nm. (b) Green emission from NBD C6-ceramide, excitation wavelength = 488 nm, scan range = 500-540 nm. (c) Overlay of the red and green channels.

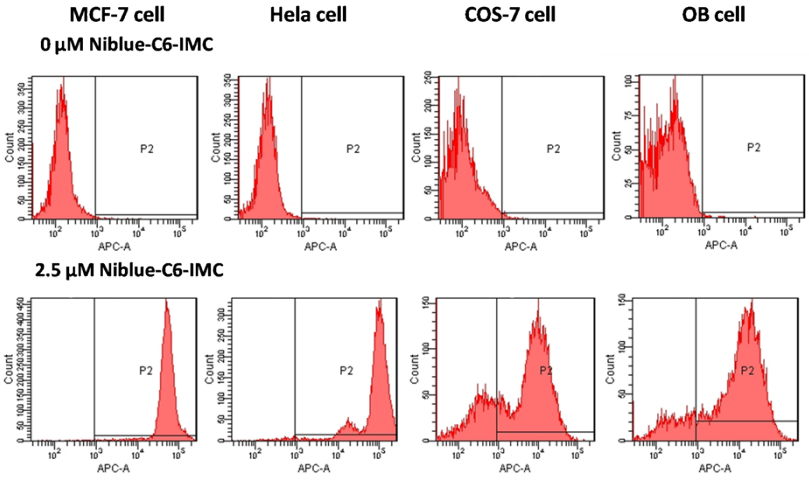


Fig. S6 Analysis of **Niblue-C6-IMC** labeled cancer cells lines (MCF-7 and Hela cells) and non-cancer cells lines (COS-7 and OB cells) by flow cytometry (λ_{ex} = 633 nm, λ_{em} = 670 nm).

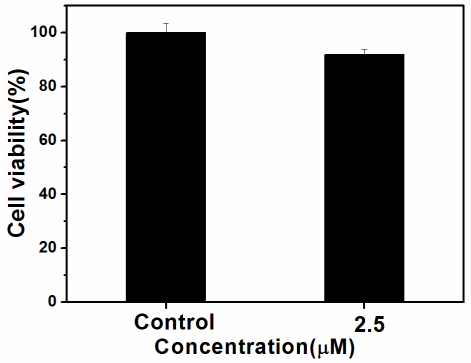


Fig. S7 Biological toxicity of **Niblue-C6-IMC** in living MCF-7 cells for 24 h.

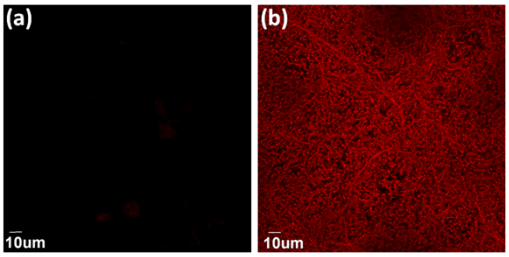


Fig. S8 Fluorescent images of **Niblue-C6-IMC** in live tissues. (a) Normal tissue slices and (b) Tumor tissue slices excised from nude mice treated with 100 μM **Niblue-C6-IMC**. After 30 min, images were obtained using excitation by OLYMPUSFV-1000 inverted fluorescence microscope at 635 nm and were collected at 640-700 nm.

^{1}H-NMR, ^{13}C-NMR, and TOF-MS spectra

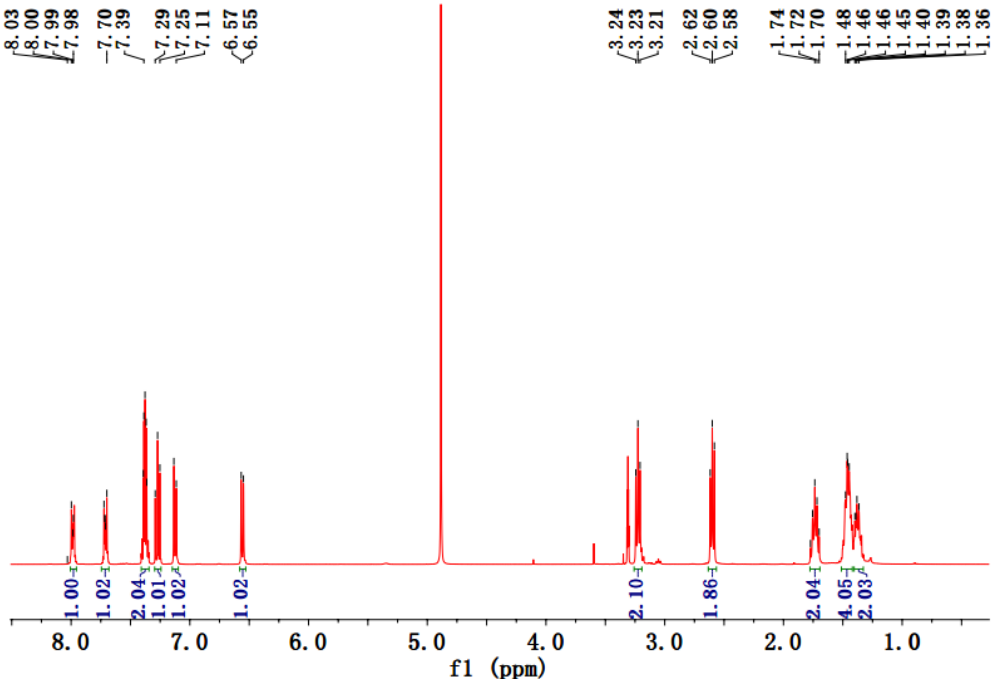


Fig. S9 ^{1}H NMR spectrum of compound **2** recorded in CD_3OD

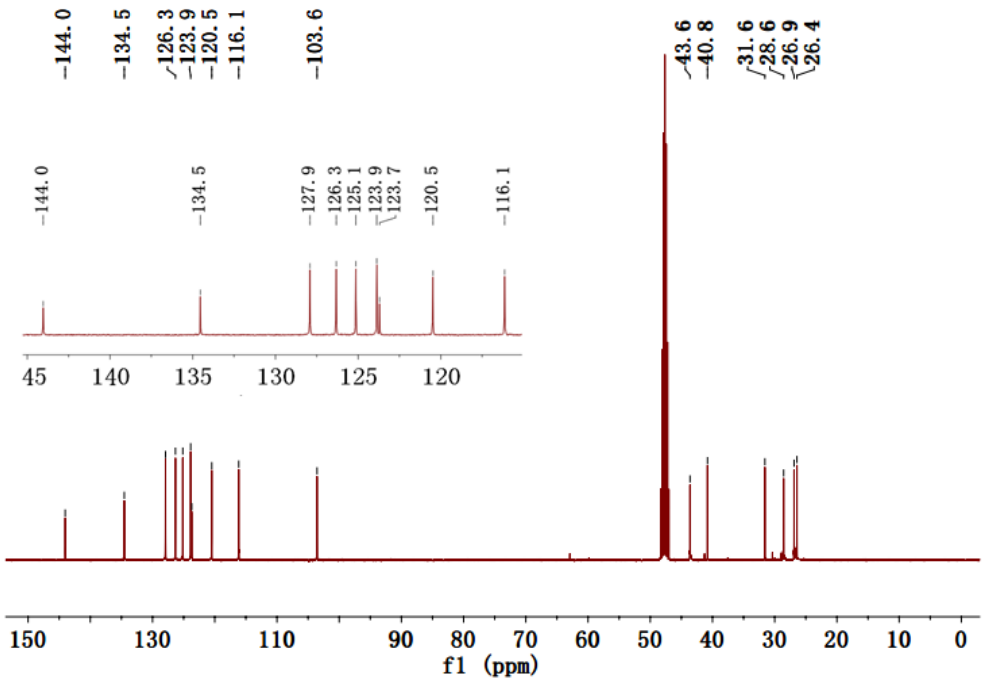


Fig. S10 ^{13}C NMR spectrum of compound **2** recorded in CD_3OD

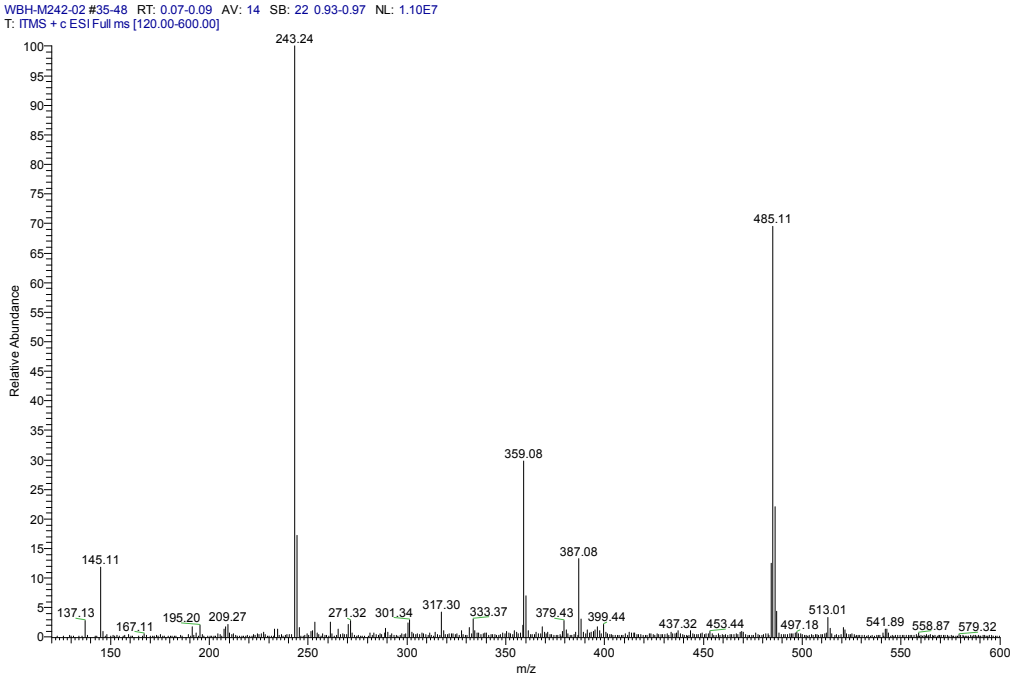


Fig. S11 TOF mass of compound **2**

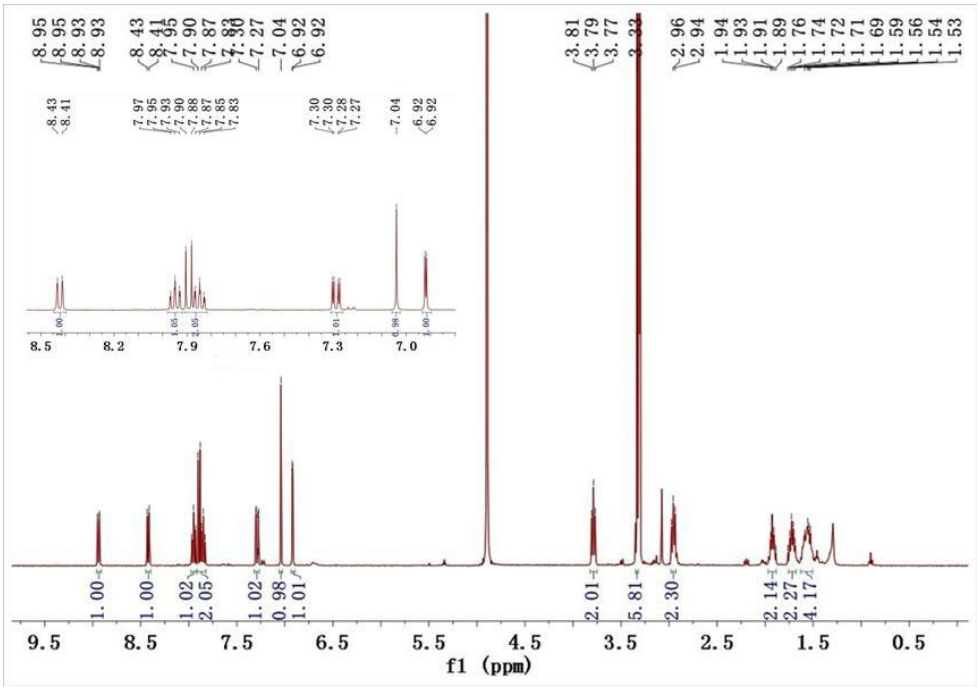


Fig. S12 ^{1}H NMR spectrum of compound **3** recorded in CD_3OD

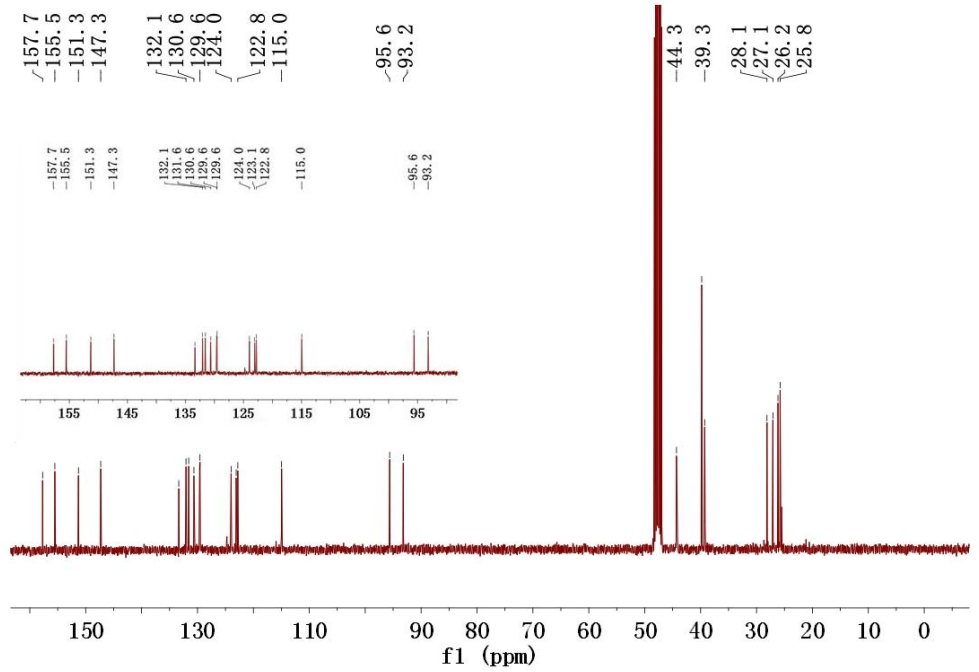


Fig. S13 ^{13}C NMR spectrum of compound **3** recorded in CD_3OD

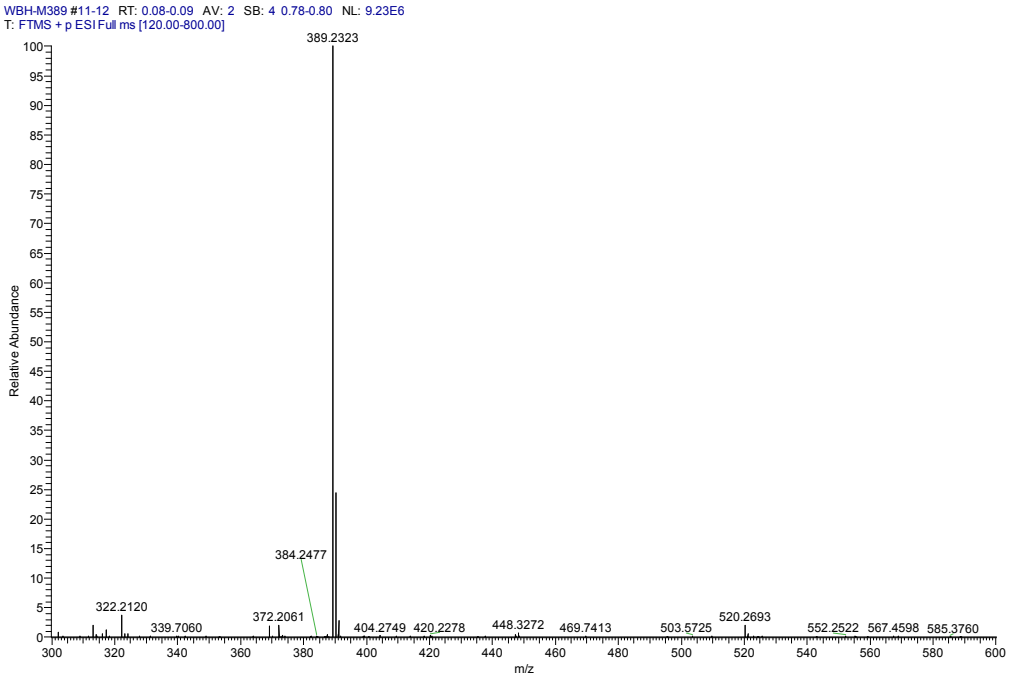


Fig. S14 TOF mass of compound **3**

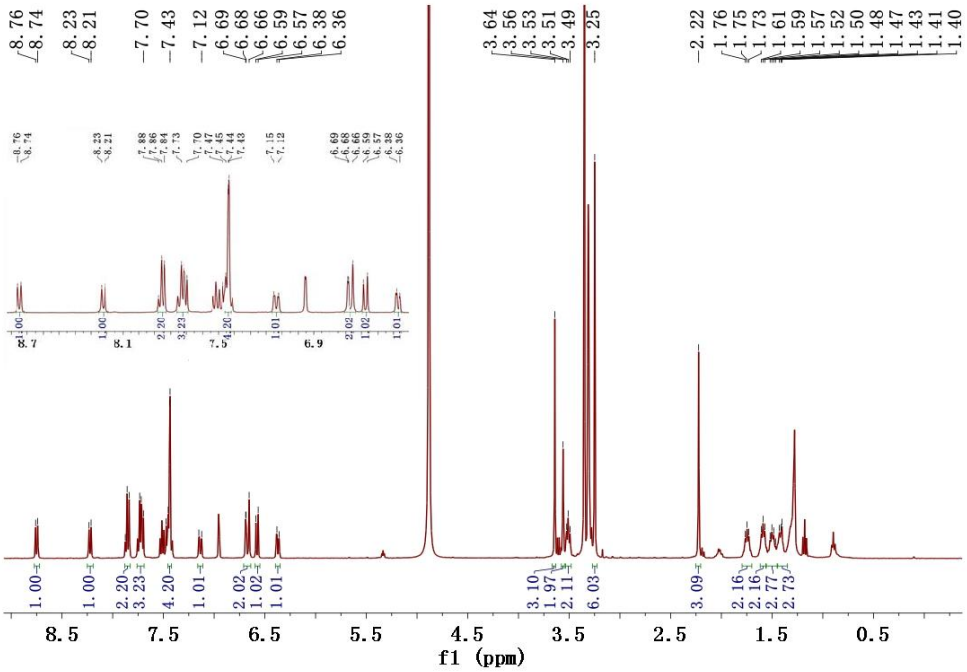


Fig. S15 ^{1}H NMR spectrum of **Niblue-C6-IMC** recorded in CD_3OD

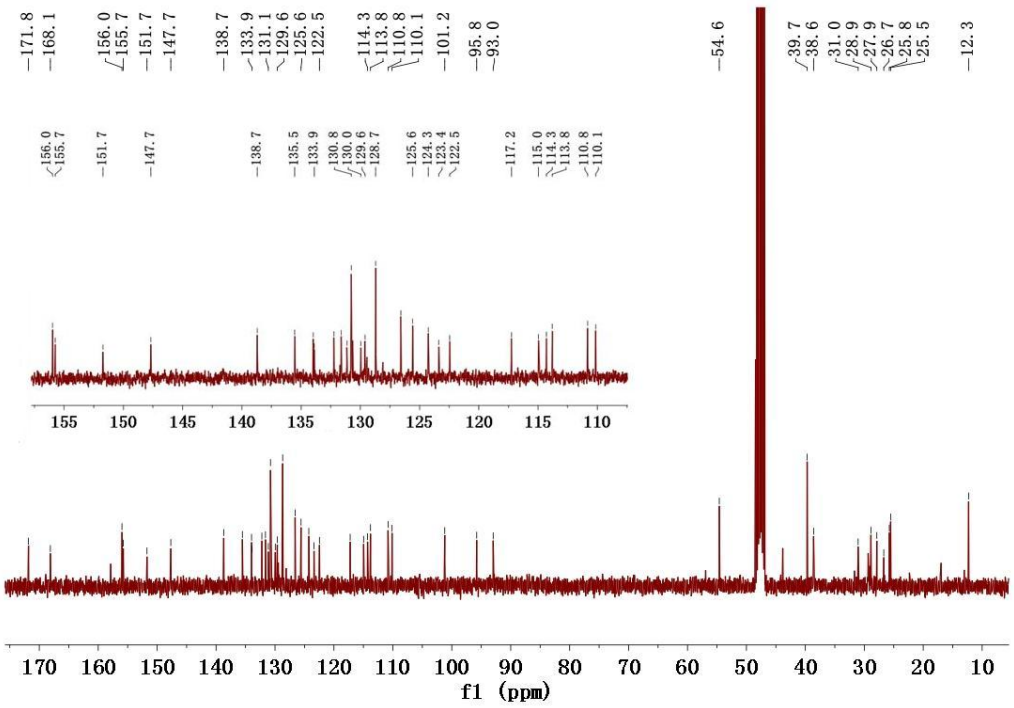


Fig. S16 ^{13}C NMR spectrum of **Niblue-C6-IMC** recorded in CD_3OD

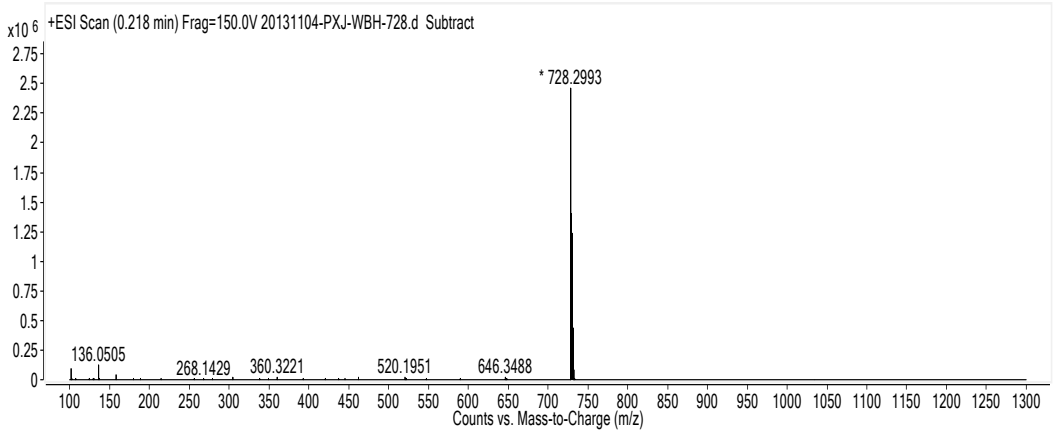


Fig. S17 TOF mass of compound **Niblue-C6-IMC**

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