Electronic Supplementary Material (ESI) for ChemComm.
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Supporting Information

Lighting-up breast cancer cells by near-infrared fluorescent probe based on KIAA1363 Enzyme-Targeting

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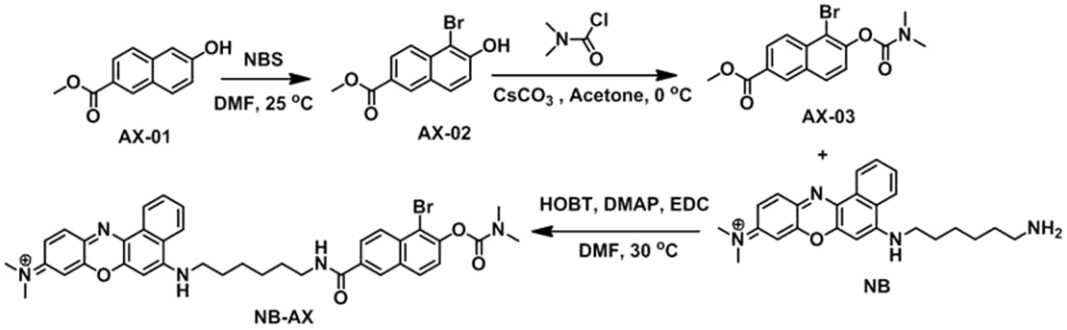
Materials and methods

All solvents and reagents used were reagent grade, and were used without further purification. The solution of **NB-AX** was dissolved in dimethyl sulphoxide (DMSO) at a concentration of 5 mM as the stock solution.^{1}H NMR and ^{13}C NMR spectra were recorded on a VARIAN INOVA-400 MHz spectrometer. Chemical shifts (δ) were reported as ppm (in DMSO, $CDCl_3$ or CD_3OD, with TMS as the internal standard). Mass spectrometric data werecarried out using HRMS instruments. Absorption spectra were measured on a HP8453 spectrophotometer (Agilent, USA). Fluorescence spectra were obtained with a FP6500 fluorescence spectrophotometer (Jasco, Japan). The relative fluorescence quantum yields of **NB-AX** in different kinds of solution were determined by Absolute fluorescence quantum yield meter (C11347-11, HAMAMATSU, Japan). ER Tracker Green, NBD-C6-ceramide, and SYTO®9 were purchased from Life Technologies Co. (USA). Recombinant Human AADACL1 protein was obtained from Abcam (ab1328620). Fluorescent responses to KIAA1363 were measured by Multiscan Spectrum (Varioskan LUX, Thermo, USA). Frozen tissue sections prepared by Leica CM1860 UV (Germany). All pH measurements were performed at room temperature (25 ± 2 ℃) using a Model PHS-3C meter calibrated with standard buffers of pH 6.86 and 9.18. Mice with tumors (MDA-MB-231) were purchased from Slac Laboratory Animal Co. (Shanghai, China).

Synthesis of NB-AX



Scheme S1. Synthetic procedures of **NB-AX** and its intermediates.

Preparation of AX-02

AX-01 (methyl 6-hydroxy-2-naphthoate) (10 mmol) and NBS were dissolved in DMF (10 ml). The mixture was stirred at 25 ℃ for 24 h with nitrogen protection. Then pour it into ice water and stand, filter to get the solid. The crude product was purified by silica gel column chromatography with CH_2Cl_2/CH_3OH and gained the white solid (AX-02) (60.5%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.53 (s, 1H), 8.13 (d, *J* = 8.9 Hz, 1H), 8.05 (d, *J* = 8.9 Hz, 1H), 7.85 (d, *J* = 8.9 Hz, 1H), 7.32 (d, *J* = 8.9 Hz, 1H), 6.13 (s, 1H), 3.98 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 166.93, 152.59, 134.72, 131.23, 130.79, 128.75, 127.34, 125.87, 125.66, 118.07, 106.15, 52.27 ppm; HRMS: m/z calcd for $C_{12}H_9BrO_3$ $[M-H]^-$: 278.9735, found: 278.9672.

Preparation of AX-03

AX-02 (3 mmol) and dimethylcarbamic chloride (10.5 mmol) and $CsCO_3$ (6 mmol) were dissolved in acetone (20 ml). The mixture was stirred at 0 ℃ for 30 h with nitrogen protection. Then wipe off the solvent with decompression spin steaming and get crude product. The crude product was purified by silica gel column chromatography with CH_2Cl_2/CH_3OH and gets the faint yellow solid (82%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.59 (s, 1H), 8.29 (d, *J* = 8.9 Hz, 1H), 8.16 (d, *J* = 8.9 Hz, 1H), 7.92 (d, *J* = 8.8 Hz, 1H), 7.43 (d, *J* = 8.8 Hz, 1H), 3.99 (s, 3H), 3.24 (s, 3H), 3.07 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 166.75, 153.59, 148.89, 134.96, 131.33, 131.08, 129.85, 127.72, 127.36, 126.96, 123.52, 115.14, 52.37, 36.94, 36.70 ppm; HRMS: m/z calcd for $C_{15}H_{14}BrNO_4$ $[M+H]^+$: 352,0106, found: $[M+H]^+$: 352.0184 and $[M+Na]^+$: 375.9981.

Preparation of NB

NB was synthesized from 5-isopropyl-2-nitrosophenol and N-(naphthalen-1-yl) hexane-1,6-diamine by the procedure published in literature.[1] ^{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, 39.3, 39.8, 44.3, 95.6, 93.2, 115.0, 122.8, 123.1, 124.0, 129.6, 129.9, 130.6, 131.6, 132.1, 133.3, 147.3, 151.3, 155.5, 157.7 ppm; HRMS: m/z calcd for $C24H29N4O^+$ $[M]^+$: 389.2336, found: 389.2323.

Preparation of NB-AX

AX-03 (1.6 mmol), NB (1 mmol), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) (2 mmol), 4-dimethylaminopyridine (DMAP) (2 mmol), 1-hydraxybenzotriazale (HOBt) (2 mmol) were dissolved in DMF (20 ml) in two round bottom flask (100 ml). The mixture was stirred at 30 ℃ for 28 h with nitrogen protection. Then wipe off the solvent with reduced pressure distillation and get crude product. The crude product was purified by silica gel column chromatography with CH_2Cl_2/CH_3OH and gets the blue solid (31.6%). ^{1}H NMR (400 MHz, MeOD) δ 8.84 (d, *J* = 8.0 Hz, 1H), 8.35 (d, *J* = 8.1 Hz, 1H), 8.23 (s, 1H), 8.14-8.06 (m, 1H), 7.91 (t, *J* = 7.5 Hz, 1H), 7.83 (dd, *J* = 18.4, 9.1 Hz, 4H), 7.36 (d, *J* = 8.8 Hz, 1H), 7.22 (d, *J* = 9.4 Hz, 1H), 6.93 (s, 1H), 6.76 (s, 1H), 3.78 (t, *J* = 7.0 Hz, 2H), 3.66 (s, 3H), 3.48 (t, *J* = 6.8 Hz, 2H), 3.28 (d, *J* = 8.8 Hz, 6H), 3.07 (s, 3H), 2.00-1.94 (m, 2H), 1.80-1.74 (m, 2H), 1.62 (m, 4H). ^{13}C NMR (100 MHz, DMSO) δ 165.52, 154.89, 154.02, 152.87, 152.06, 148.91, 148.01, 147.33, 145.70, 141.51, 132.84, 132.46, 131.03, 130.14, 129.74, 129.60, 129.44, 127.81, 126.38, 126.13, 124.22, 123.97, 123.76, 123.20, 114.20, 109.20, 96.75, 95.83, 50.27, 38.58, 36.44, 36.26, 30.68, 29.09, 26.88, 26.43 ppm; HRMS: m/z calcd for $C_{38}H_{39}BrN_5O_4^+$ $[M]^+$: 708.2185, found: 708.2175.

Photophysical properties of NB-AX

Absorption UV-visible spectra were collected on a HP8453 spectrophotometer (Agilent, USA). Fluorescence spectra were obtained with a FP6500 spectrophotometer (Jasco, Japan) with slit widths set at 2.5 nm and 2.5 nm for excitation and emission, respectively. The relative fluorescence quantum yields were determined by Absolute fluorescent quantum yield meter.

Quantitative detection of KIAA1363 in vitro

Fluorescent responses to KIAA1363 were measured on Multiscan Spectrum at 650-830 nm by 630±12 stimulating at 37 ℃.

KIAA1363 IC50 assay

Inhibitor (NB-AX) and substrate (4-MUBA, 250 mM) were added to each well of a 96-well, followed by addition of the serine hydrolase (KIAA1363) to initiate the hydrolytic reaction (37 ℃).Fluorescence intensity of catalysate with increased concentration of Inhibitor (NB-AX) was detected (λex =302 nm, λem =356 nm).

Determination of the detection limit

The detection limit was calculated based on the fluorescence titration curve (Figure 1b) of **NB-AX** in the presence of KIAA1363 (0-7.2 g/ml). The fluorescence intensity of **NB-AX** was measured three times and the standard deviation of blank measurement was achieved. The detection limit was calculated with the following equation:

Detection limit = 3σ/k. Where σ is the standard deviation of blank measurement, k is the slop between the fluorescence intensity versus KIAA1363 concentrations.

The quantum yield of NB-AX with KIAA1363

The quantum yields of NB-AX with KIAA1363 in Tris-HCl buffer (pH 8.0) were determined according to the method below :

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

Where φ is fluorescence quantum yield; FA is integrated area under the corrected emission spectra; A is the absorbance at the excitation wavelength; λex 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 0.89 (λex=495 nm) in ethanol.

Cell incubation and staining with NB-AX

The mammalian cells MCF-7, MDA-MB-231, T47D (human breast cancer cells), MCF-10A (normal breast epithelial cells), RWPE-1 (human normal prostate epithelial cells) and LO-2(human normal liver cells) 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 and 1% gentamicin sulphate. The cells were seeded in 24-well flat-bottomed plates and incubated for 24 h at 37 ℃ under 5% CO_2. Before imaging, the live cells were incubated with 2.5 μM NB-AX for another 10 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. The stain images were obtained using excitation and emission wavelengths at 635 nm and 655-755 nm, respectively.

Fluorescence counterstaining of live cells

SYTO®9 (1.0 μM), ER-Tracker Green (2.0 μM) and NBD-C6-ceramide (1.0 μM) were used to co-stain the cells. Cells were incubated for 10 min at 37 ℃ under 5% CO_2 and then washed with PBS three times. Fluorescence images were then carried out with OLYMPUSFV-1000 inverted fluorescence

microscope, using a 60×objective lens. **NB-AX** (red channel) was excited at 635 nm, and the emission spectra were collected at 655-755 nm. The commercially available probes of subcellular organelles (green channel) were excited at 488 nm, and the emission spectra were collected at 500-550 nm.

Cytotoxicity experiments

Measurement of cell viability was evaluated by MTT (3-(4, 5)-dimethylthiahiazo (-2-yl)-3, 5-diphenytetrazoliumromide) to formazan crystals using mitochondrial dehydrogenases. MCF-7 cells were seeded in 96-well microplates 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 with2.5 and 5.0 μM of **NB-AX** for 24 and 48 h, respectively. Cells in culture medium without **NB-AX** were used as the control. Five 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 37 ℃ 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[2] was expressed as a percent of the control culture value, and it was calculated using the following equation:

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

Where dye stands for the sample containing **NB-AX**, control is control group, and the blank group is denoted as K.

Flow cytometry

MCF-7, T47D, RWPE-1, LO-2 cells were cultured in DMEM supplemented with 10% FBS under an atmosphere of 5 % CO_2 at 37 ℃. 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 DMEM. After incubation at 37 ℃ 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 DMEM was added along with Lyso-NINO and/or iNOS stimulants. 10^5 cells were plated into a six-chamber culture well and incubated for 24 h. **NB-AX** was added to the culture medium, and the cells were incubated for 10 min. A 630 nm argon ion laser was used for excitation. Signals from cells were collected at 670±25 nm. Cells were analyzed in a FAC Scan cytometer (Becton Dickinson Biosciences Pharmingen, USA).[3]

Preparation of tissue slices and staining with NB-AX

Tissues were prepared from breast cancer tissues and paired normal tumor-adjacent tissues. The slices were cut at 4 μm using Leica CM1860 UV at quick frozen state. Then incubated tissue slices with NB-AX (10 μM) in PBS buffer for 5 min at 37 ℃. The tissue slices were imaged using OLYMPUSFV-1000 inverted fluorescence microscope with a 635 nm excitation laser and emission spectra were collected at 655-755 nm after washing with PBS buffer three times. The tissue slices for depth imaging were cut at 1000 μm then incubated with **NB-AX** (100 μM) in 2 h.

Fluorescence imaging in vivo

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 tumourbearing mice were performed about 8 days, when implants grew up to about 0.5 cm in size. The mice with tumours were given a subcutaneous injection of **NB-AX**(200 μM/100 μL) at about 2 cm away from the tumour region, near the enterocoelia. After injected **NB-AX** 40 min, the mice were imaged using a NightOWL II LB983 small animal in vivo imaging system with 630 nm excitation laser and 700 nm emission filter.[4]

Supplementary Figures

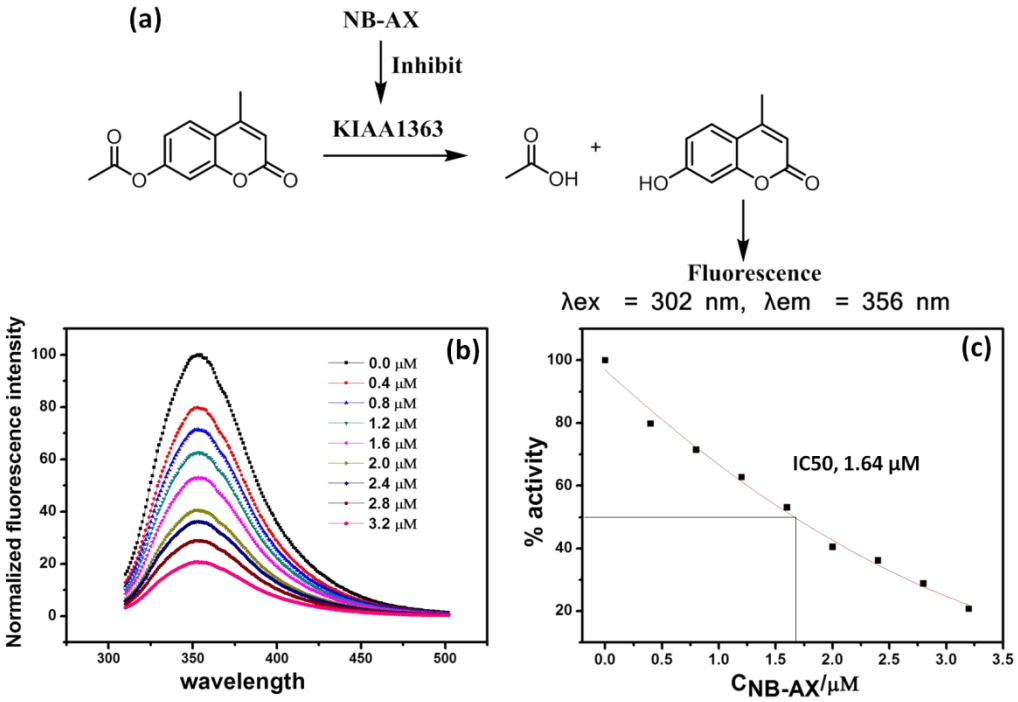


Figure S1. Measured KIAA1363 activities using fluorescence assay. (a) the hydrolysis reaction catalyzed by recombinant serine hydrolase. (b) Inhibition of KIAA1363 by varying concentrations of NB-AX (0-3.2 μM). The progress of the 4-MUBA hydrolysis reaction was followed by measuring the fluorescence of 4-methylumbelliferone produced (λex = 302 nm, λem = 356 nm) for 5 min at 37 ℃. (c) Fluorescence at 356 nm responses to the KIAA1363 concentration (0-3.2 μM).

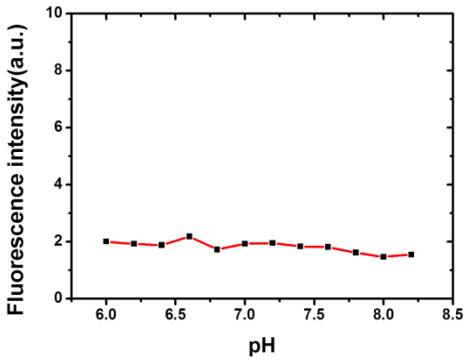


Figure S2. Effect of pH value from 6.0 to 8.2 on the fluorescent intensity of **NB-AX** (5 μM).

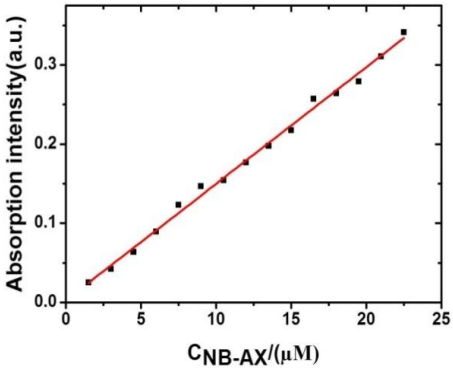


Figure S3. The water solubility of **NB-AX** (R^2=0.98).

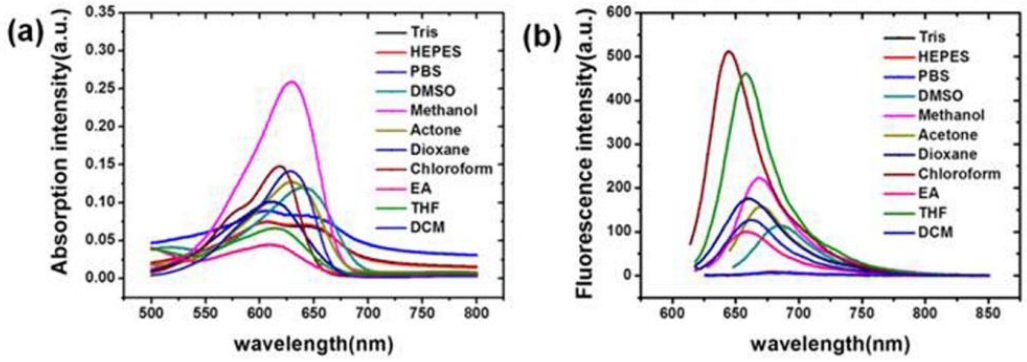


Figure S4. The absorbance (a) and emission (b) spectra of **NB-AX** (5 μM) in different solvents.

Table S1. Photophysical properties of **NB-AX** in various solvents

Solvent	λ_{abs}(nm)	λ_{em}(nm)	$\triangle\lambda$(nm)	ε(10^5 L/(M^{-1}*cm))	Φ
Tris-HCl	629	685	77	0.147	0.023
HEPES	633	685	77	0.149	0.024
PBS	638	685	77	0.178	0.022
DMSO	642	685	43	0.240	0.236
Methanol	629	667	38	0.519	0.216
Acetone	630	670	40	0.255	0.252
1,4-Dioxane	612	660	48	0.203	0.375
Chloroform	618	644	26	0.395	0.648
EA	610	657	47	0.089	0.345
THF	613	661	48	0.132	0.402
DCM	630	657	27	0.281	0.588

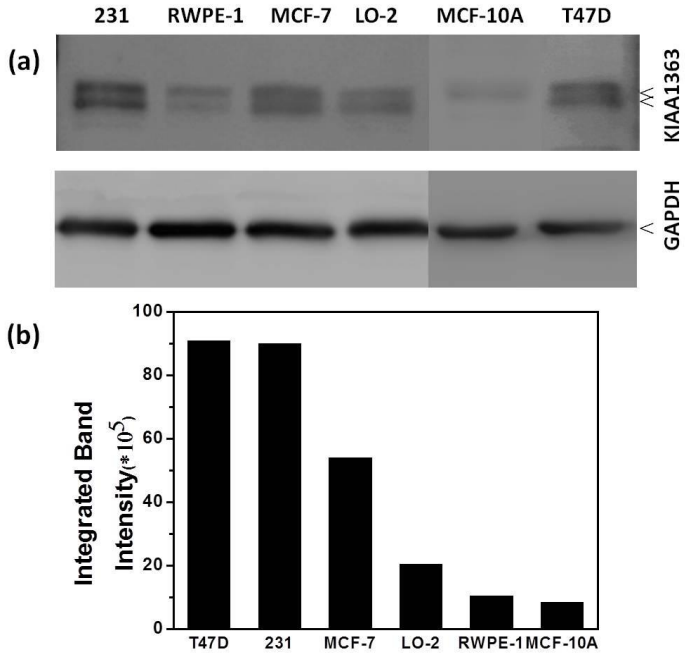


Figure S5. (a) KIAA1363 activity abundance in lysate of T47D cells, MCF-7 cells, MDA-MB-231 cells, MCF-10A cells, RWPE-1 cells and LO-2 cells. (b) Quantitative analysis of KIAA133 activity abundance intensity. Integrated Band Intensity = the gray value of KIAA1363 bands / the gray value of GAPDH bands. The gray values were read by Quantity One.

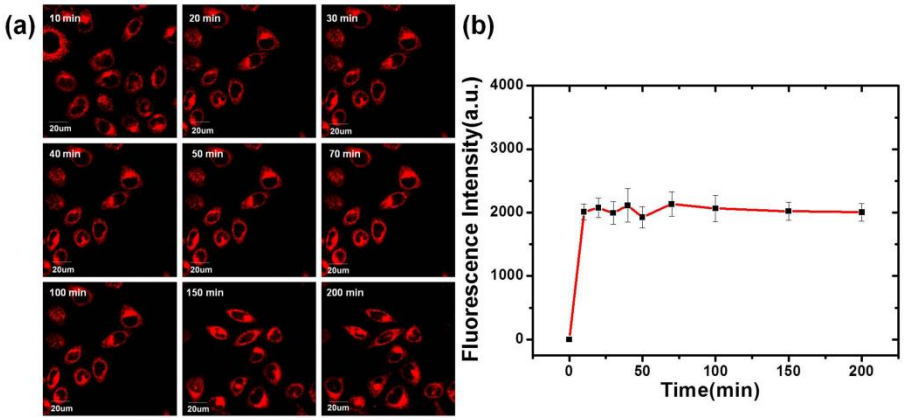


Figure S6. Real-time (0-200 min) fluorescence images of **NB-AX** in live MCF-7 cells. (a) cells were cultured with 2.5 μM NB-AX and then confocal fluorescence images were recorded at different time points, excitation wavelength = 630 nm; scan range = 655-755 nm; (b) Quantitative image analysis of the average fluorescence of cells, determined from analysis of 7 areas in each sample image.

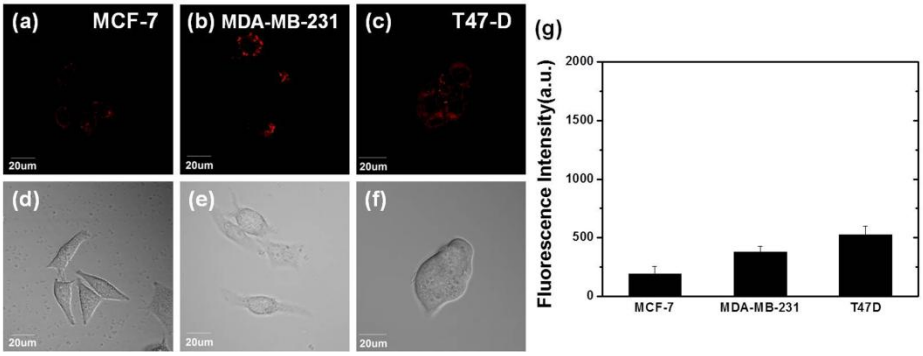


Figure S7. Living cells staining with JW480 (7.5 μM) for 3 h then NB-AX (2.5 μM) for 10 min. (a), (b) (c) are fluorescent images and (d), (e) (f) are bright-field images. Excitation wavelength = 630 nm, scan range = 655-755 nm; (g) Quantitative image analysis of the average fluorescence of cells, determined from analysis of 7 areas in each sample image of cells selectivity experiments.

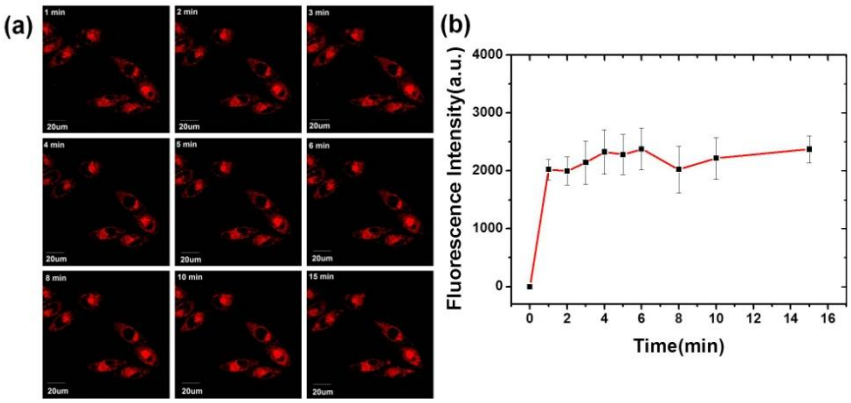


Figure S8. Real-time fluorescence images of **NB-AX** in live MCF-7 cells. (a) cells were cultured with 2.5 μM **NB-AX** and then confocal fluorescence images were recorded at different time points, excitation wavelength = 630 nm; scan range = 655-755 nm; (b) Quantitative image analysis of the average fluorescence of cells, determined from analysis of 9 areas in each sample image.

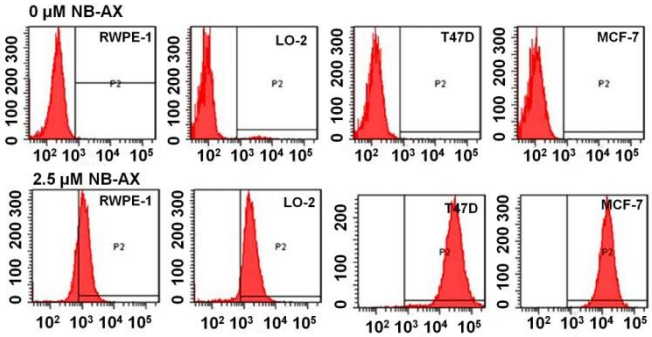


Figure S9. Analysis of **NB-AX**labeled cancer cells lines (MCF-7 and T47D cells) and non-cancer cells lines (RWPE-1 and LO-2 cells) by flow cytometry (λex = 630 nm, λem = 700 nm).

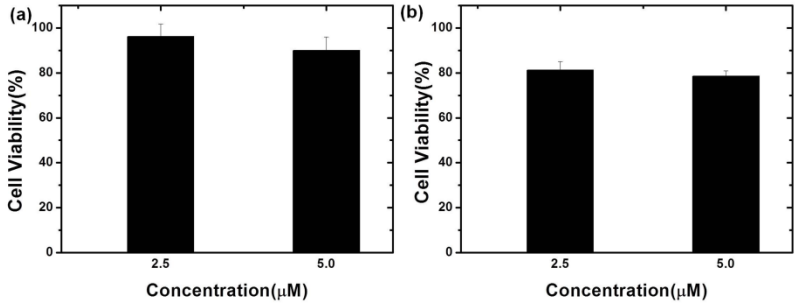


Figure S10. Cytotoxicity of **NB-AX** in living MCF-7 cells for 24 h (a), 48 h (b).

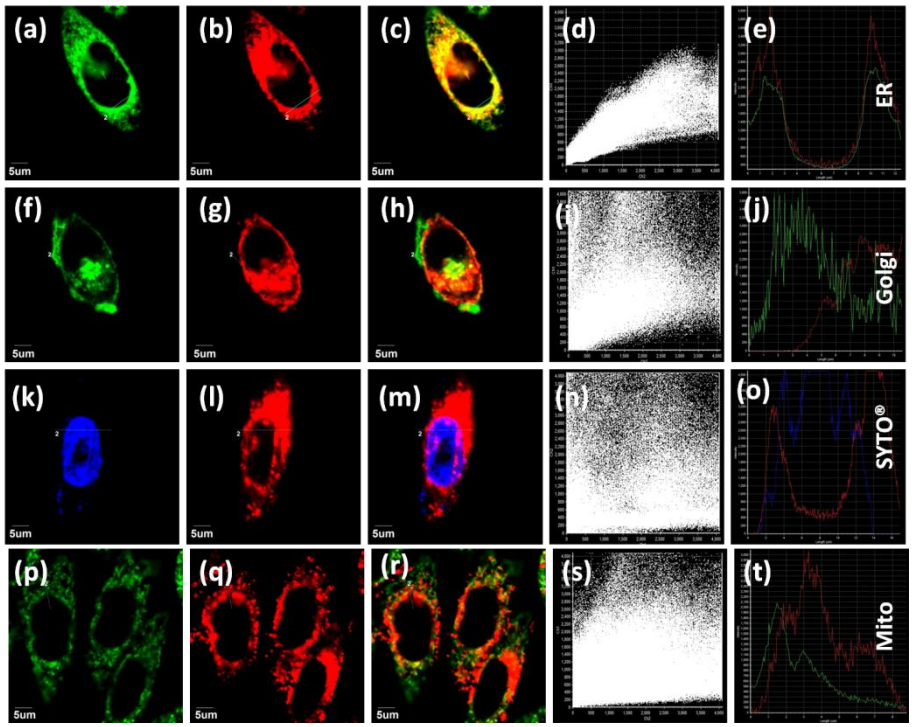


Figure S11. Fluorescence images of NB-AX (2.5 μM), ER-Tracker Green 2.0 μM, Golgi-Tracker Green 1.0 μM, SYTO®9 1.0 μM, Mito-Tracker Green 2.0μM in MCF-7 cells. (a, f, k, p) Green (or blue) channel. Excitation wavelength = 488 nm, scan range = 500-550 nm. (b, g, l,q) Red emission of NB-AX, excitation wavelength = 630 nm, scan range = 655-755 nm. (c, h, m, r) Overlay of the green (or blue) and red channel. (d, i, n, s) Co-localization analysis (Rr = 0.96, 0.70, 0.34, 0.59 respectively). (e, j, o, t) Intensity profile of region cross co-stain image.

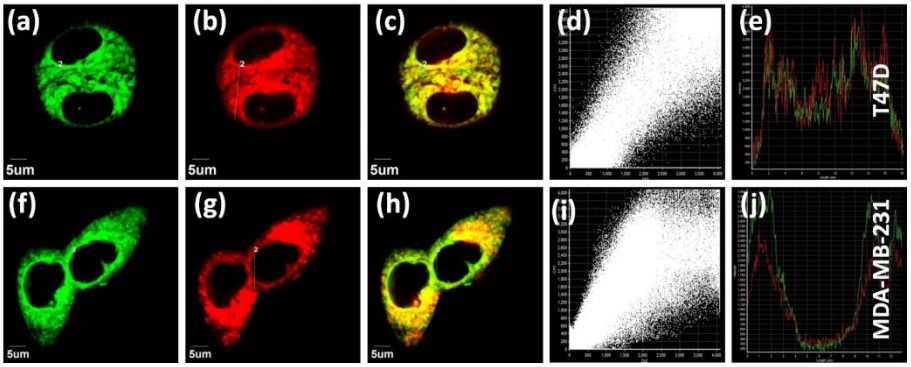


Figure S12. Fluorescence images of NB-AX (2.5 μM), ER-Tracker Green 2.0 μM in T47D and MDA-MB-231 cells. (a, f,) Green channel. Excitation wavelength = 488 nm, scan range = 500-550 nm. (b, g,) Red emission of NB-AX, excitation wavelength = 630 nm, scan range = 655-755 nm. (c, h,) Overlay of the green and red channel. (d, i,) Co-localization analysis (Rr = 0.94, 0.91 respectively). (e, j,) Intensity profile of region cross co-stain image.

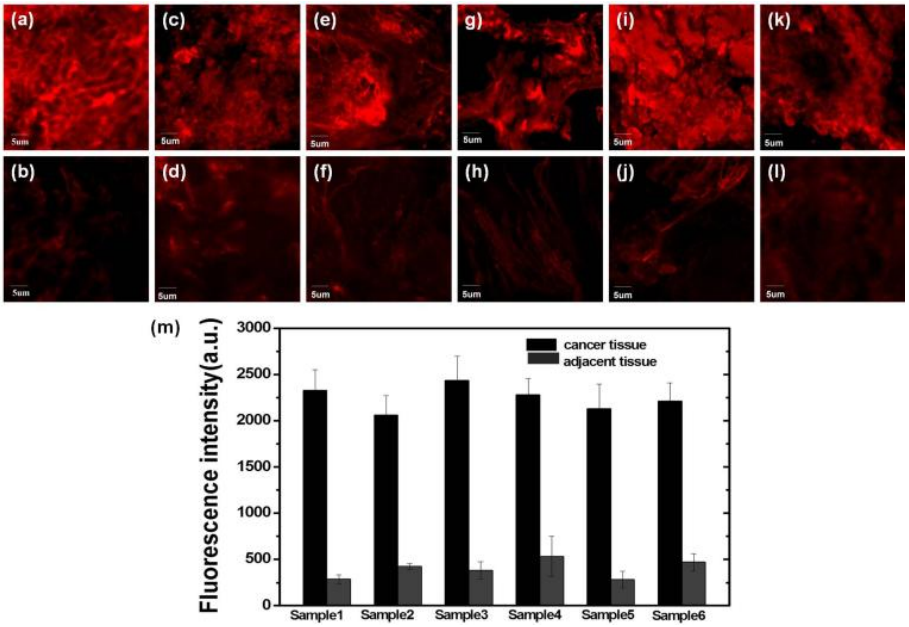


Figure S13. Fluorescent images of **NB-AX** in tissues (human breast cancer tissues (up) and adjacent tissues (down)) stained with **NB-AX** 10 μM for 5 min. (a,b), (c,d), (e,f), (g,h), (i,j) and (k,l) belong to experiments named sample 1-6 respectively. Images were generated using an excitation wavelength of 635 nmand were collected at 655-755 nm. (m). Quantitative image analysis of the average fluorescence, determined from analysis of 7 areas in each sample image.

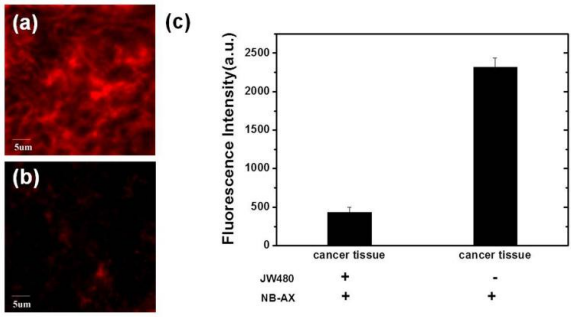


Figure S14. Competitive fluorescent images of NB-AX in tissues. (a) Cancer tissues stained with NB-AX 10 μM for 10 min, excitation wavelength = 630 nm; scan range = 655-755 nm ; (b) Cancer tissues stained with JW480 50 μM for 2 h, then stained with NB-AX 10 μM for 10 min, excitation wavelength = 630 nm; scan range = 655-755 nm. (e). Quantitative image analysis of the average fluorescence, determined from analysis of 7 areas in each sample image.Figure S14**.**In-depth imaging of **NB-AX** stained breast cancer tissue. Images were generated using an excitation wavelength of 635 nm and were collected at 655-755 nm.

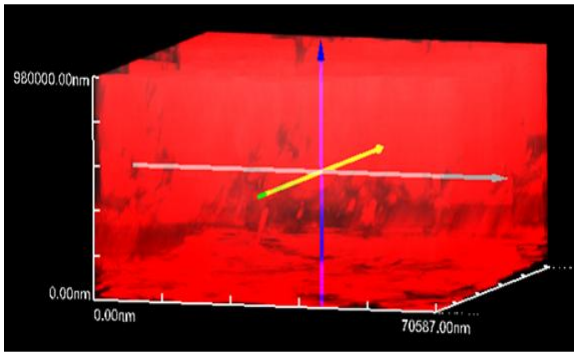


Figure S15. In-depth imaging of **NB-AX** stained breast cancer tissue. Images were generated using an excitation wavelength of 635 nm and were collected at 655-755 nm.

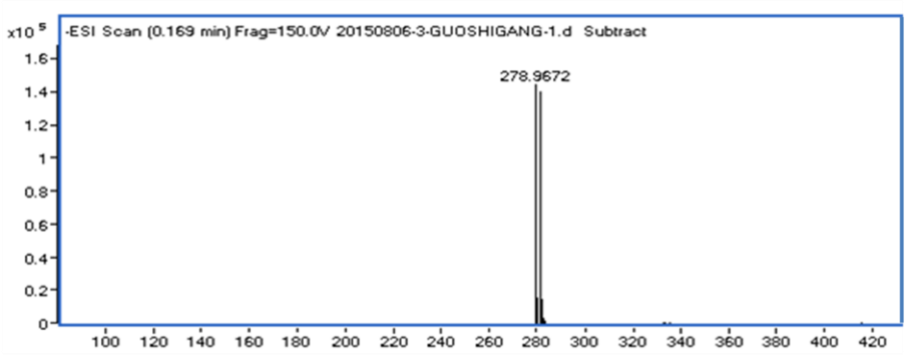


Figure S16. HRMS of AX-02.

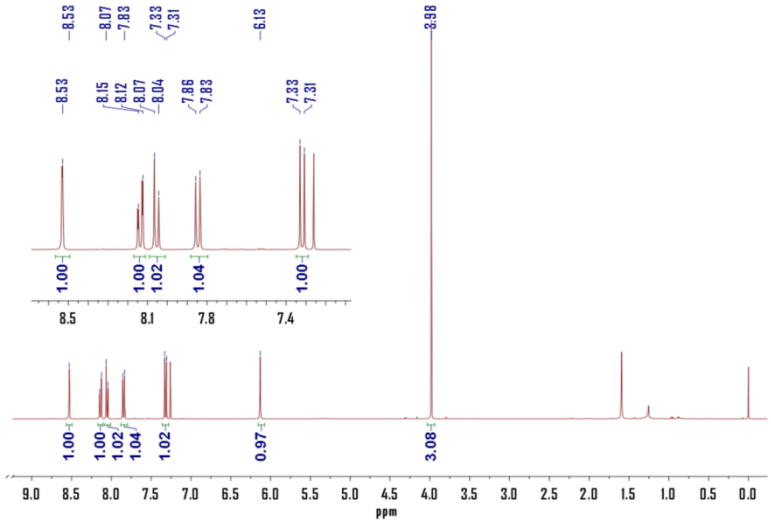


Figure S17. ^{1}H-NMR spectrum of AX-02 recorded in $CDCl_3$.

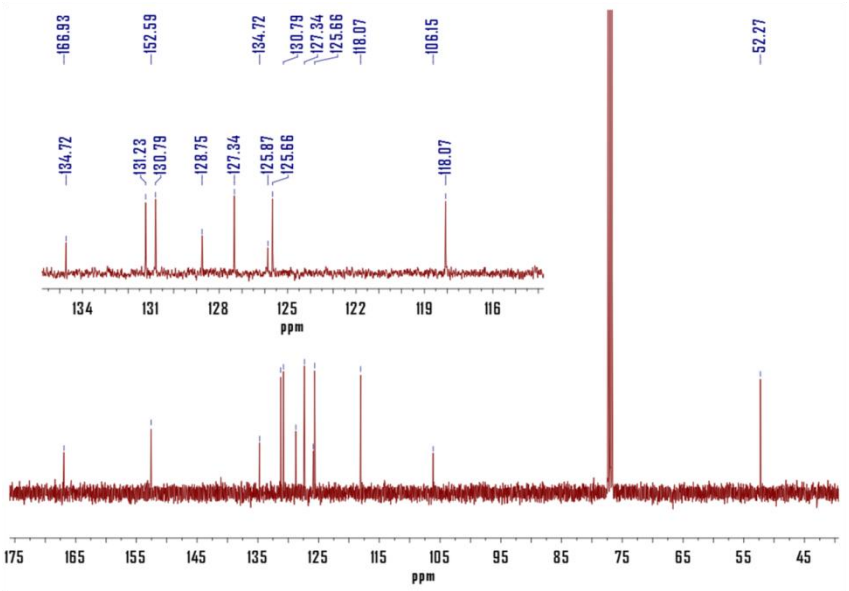


Figure S18. ^{13}C-NMR spectrum of AX-02 recorded in $CDCl_3$.

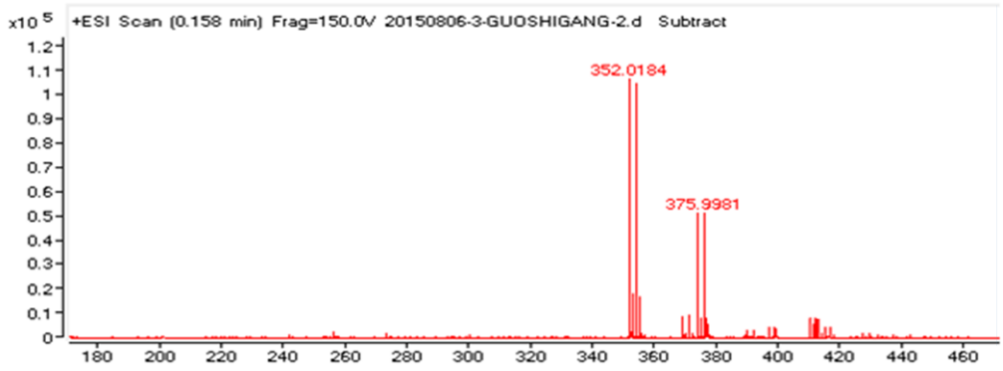


Figure S19. HRMS of AX-03

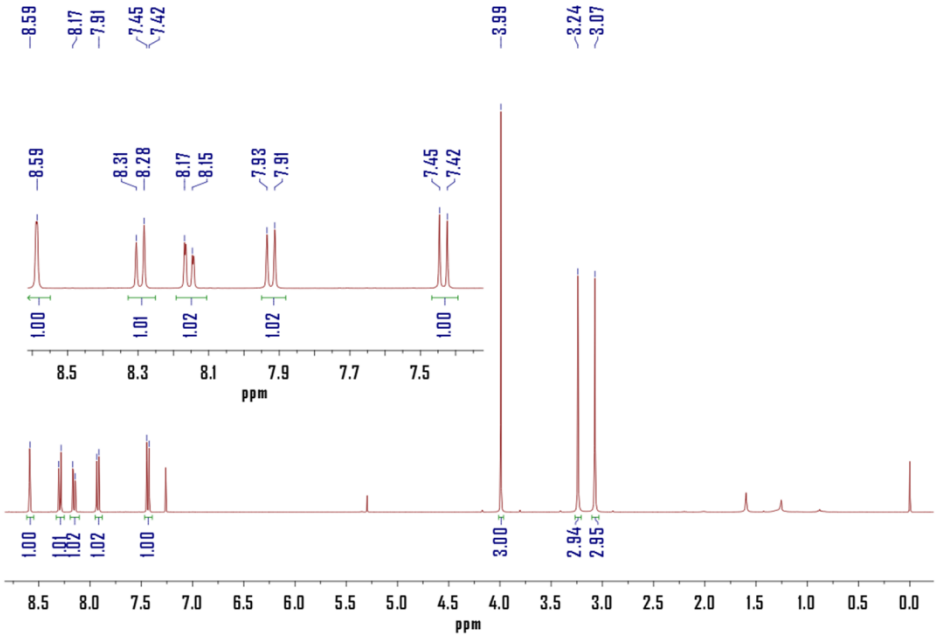


Figure S20.[1]H-NMR spectrum of AX-03 recorded in $CDCl_3$.

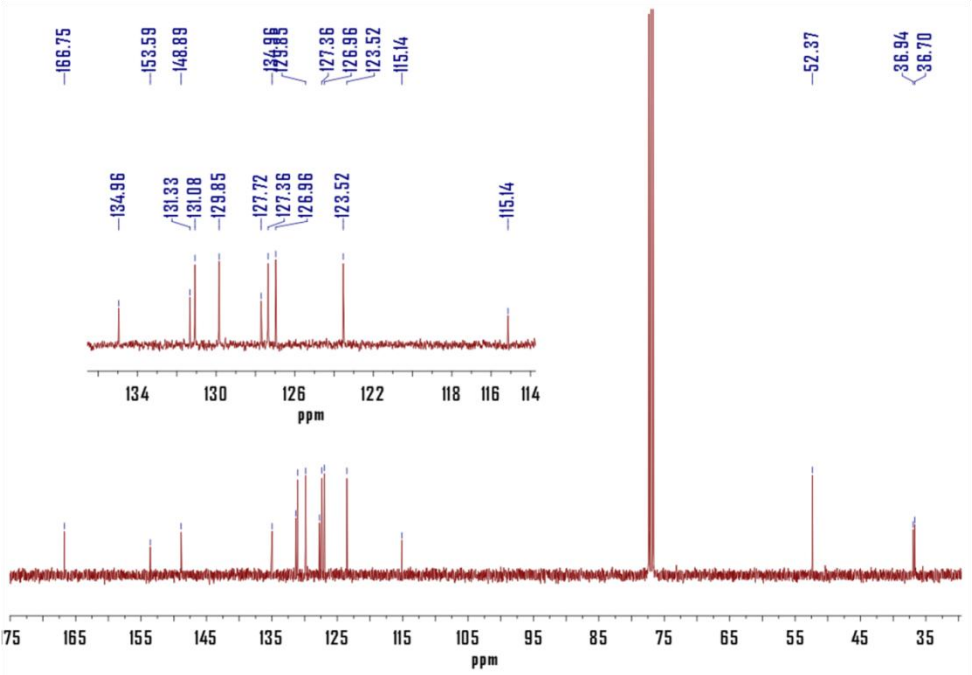


Figure S21. ^{13}C-NMR spectrum of AX-03 recorded in $CDCl_3$

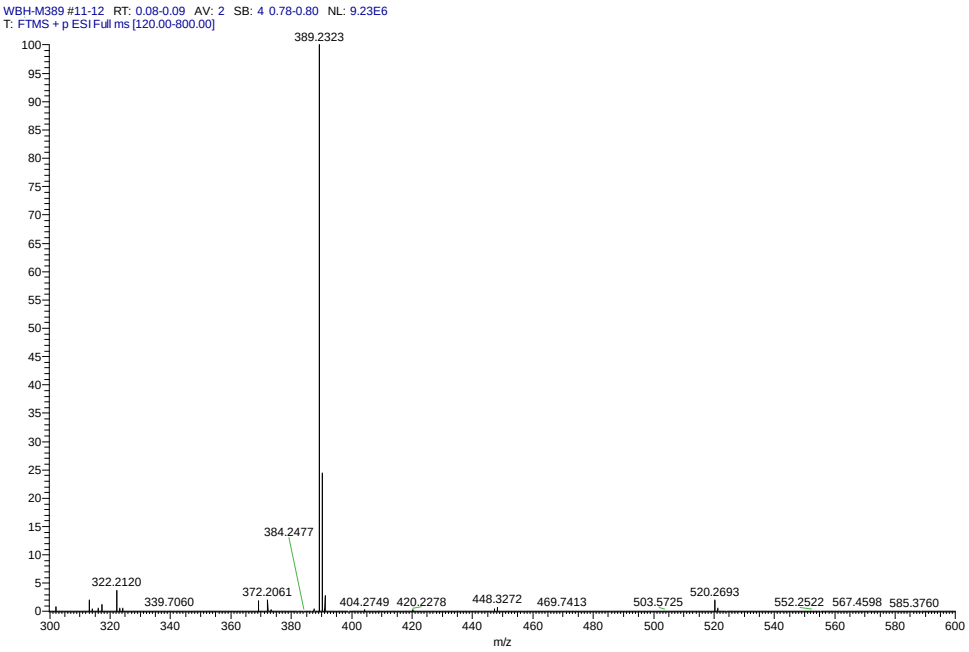


Figure S22.HRMS of NB.

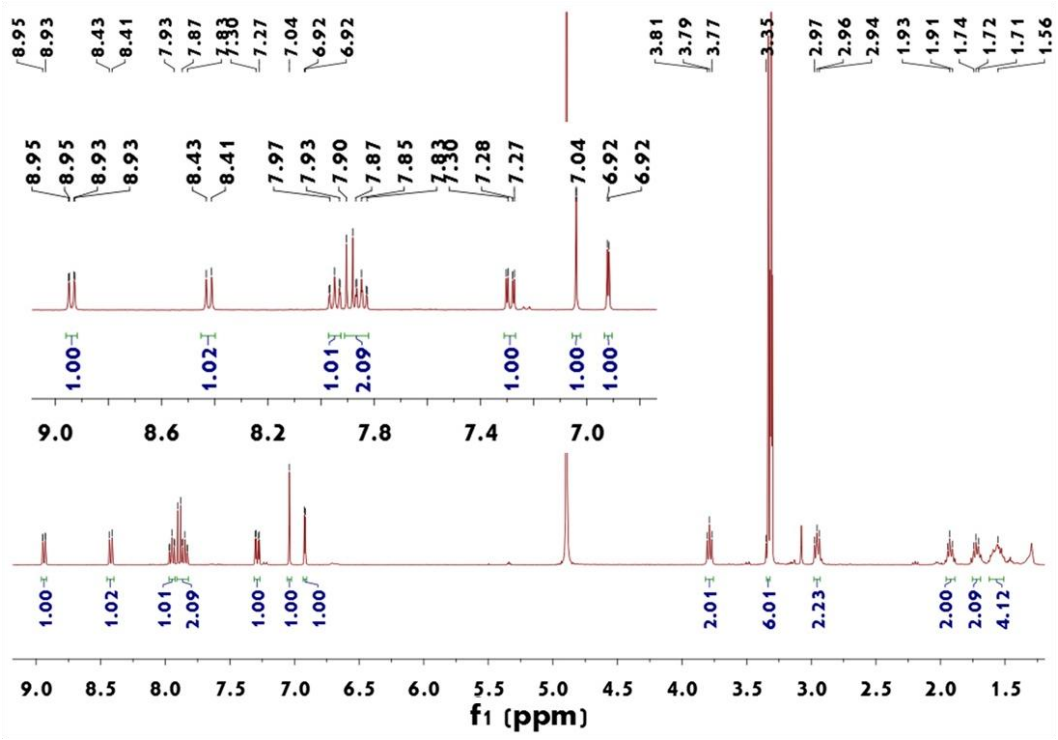


Figure S23.[1]H-NMR spectrum of NB recorded in CD_3OD.

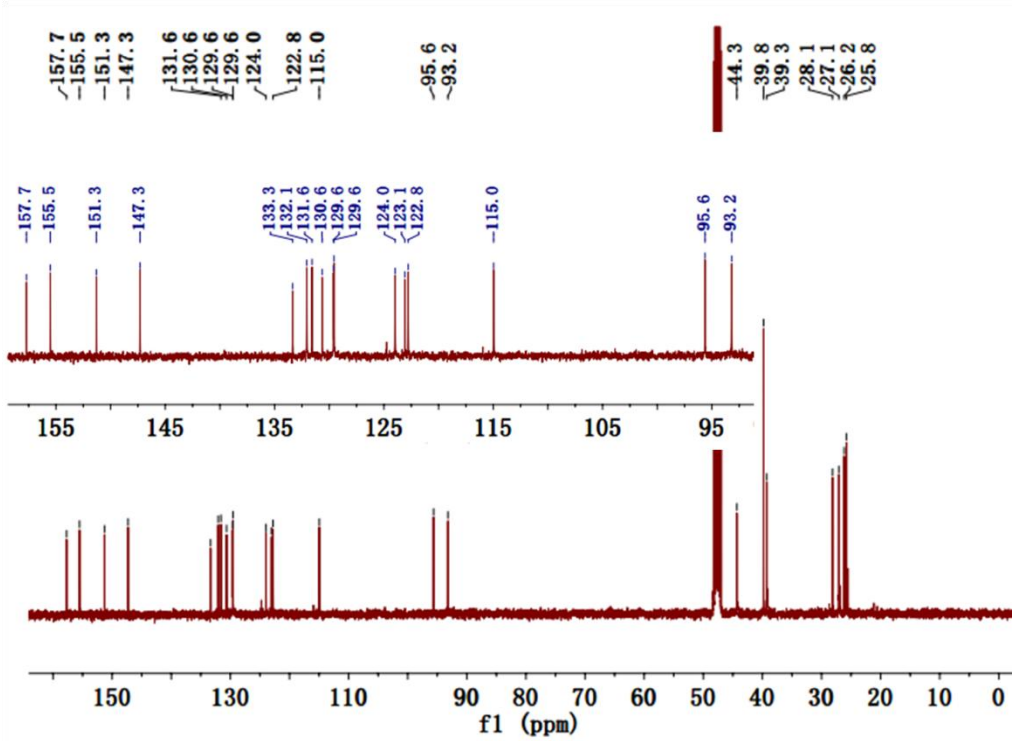


Figure S24.[13]C-NMR spectrum of NB recorded in CD_3OD

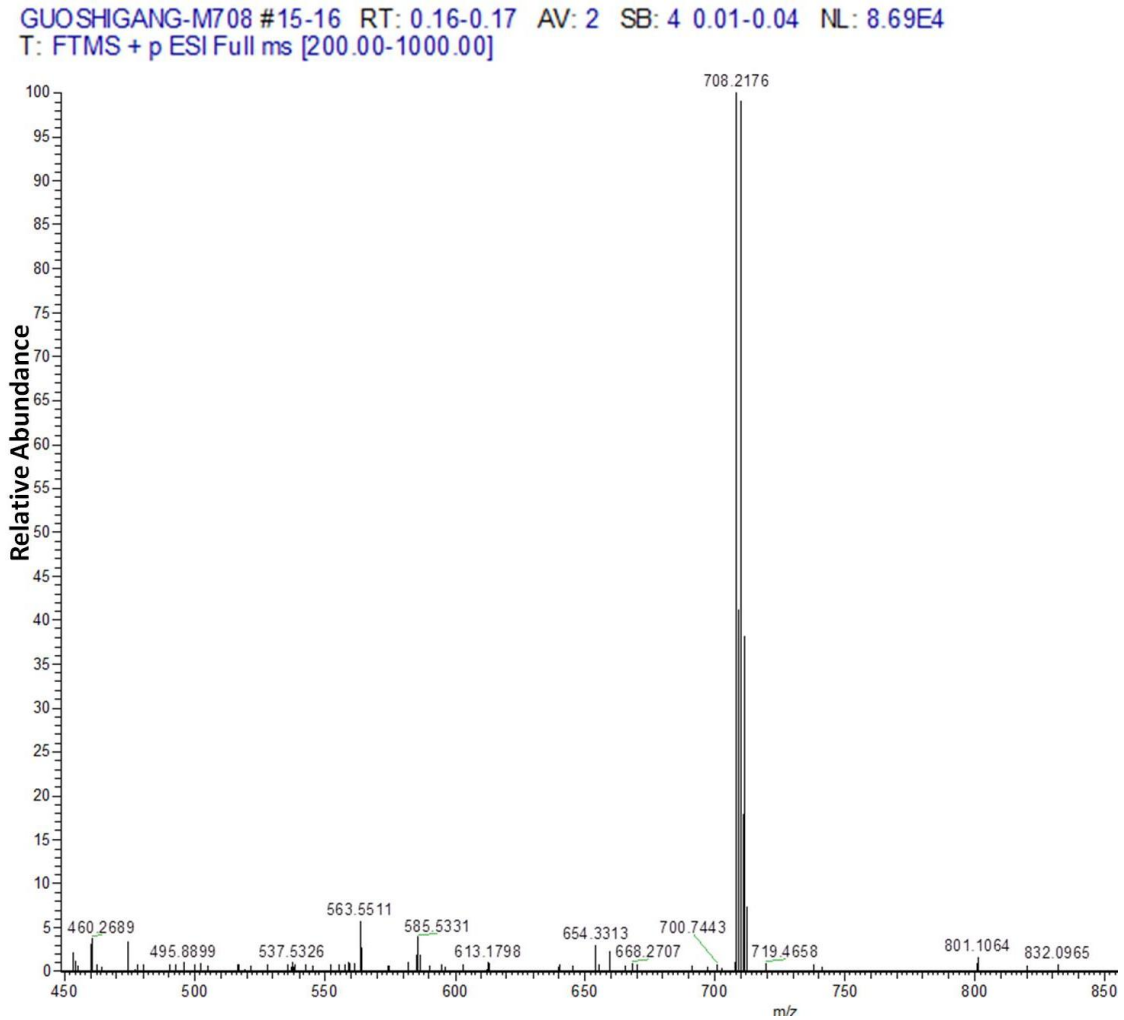


Figure S25. HRMS of NB-AX.

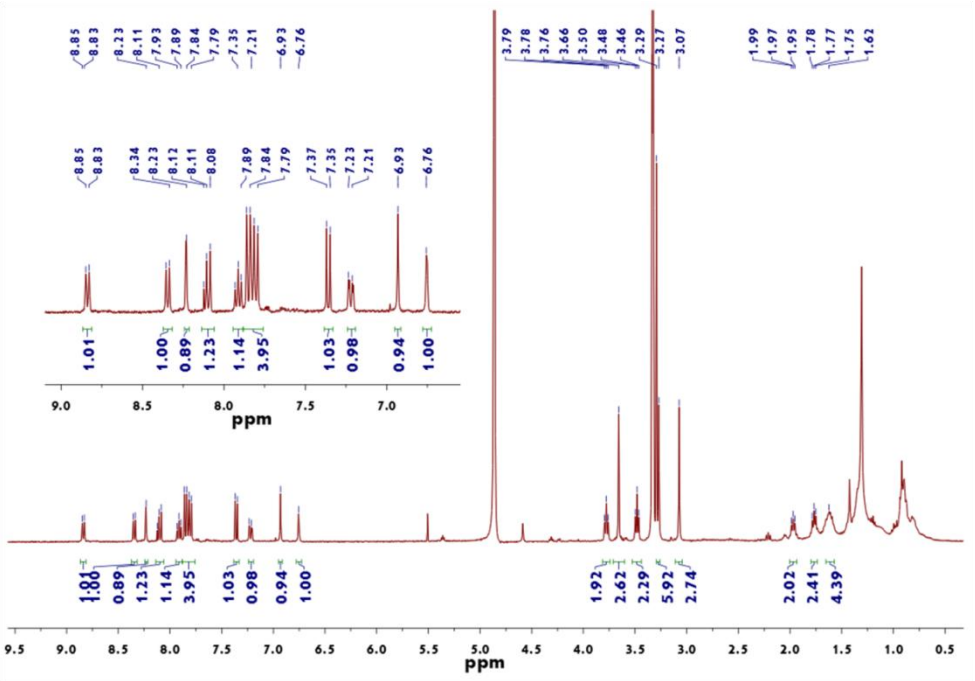


Figure S26. 1H-NMR spectrum of NB-AX recorded in CD3OD.

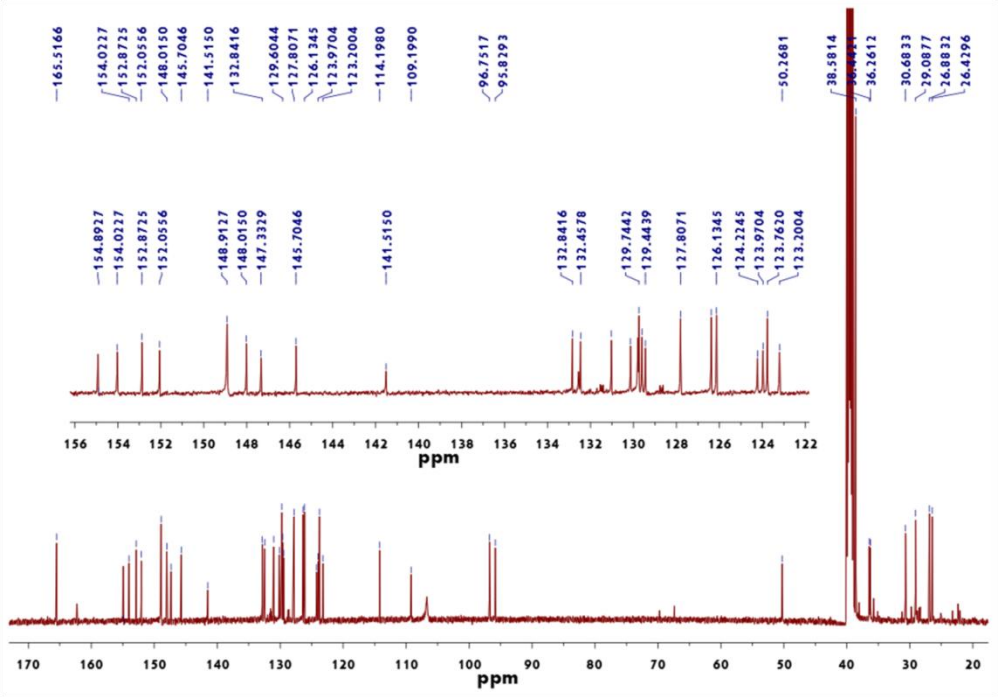


Figure S27. ^{13}C-NMR spectrum of NB-AX recorded in DMSO-d_6.

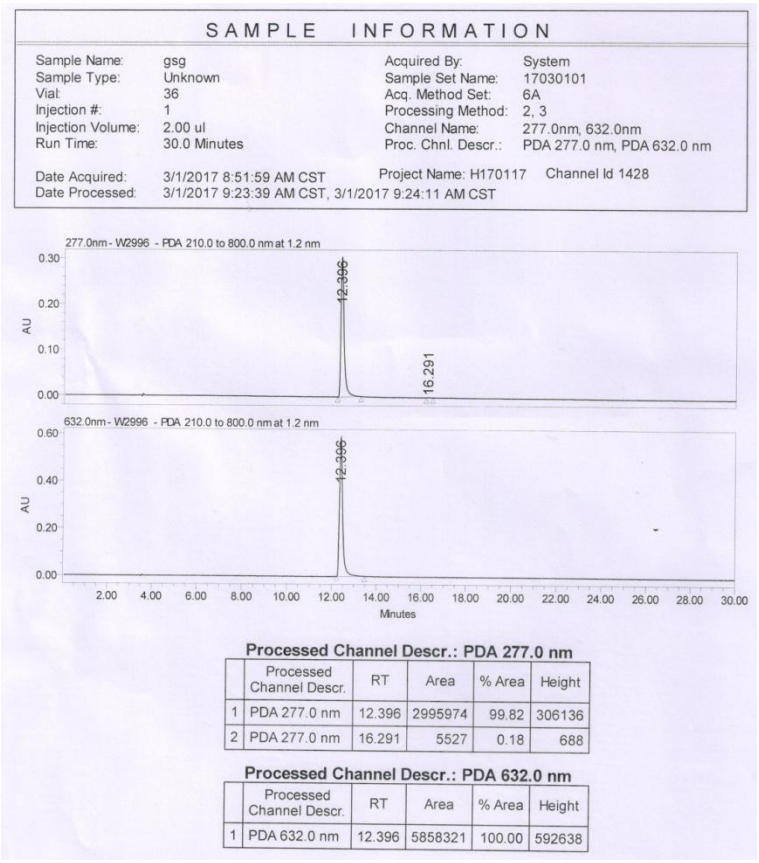


Figure S28. The HPLC trace of NB-AX.

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