Electronic Supplementary Information

Activatable Fluorescent-Photoacoustic Dual-Modal Probe for Highly Sensitive Imaging of Nitroxyl in Vivo

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1. Experimental Procedures

1.1 Reagents and Instruments

All chemicals were purchased from commercial suppliers and used without further purification. Mito-Tracker Green, Lyso-Tracker Green, Hoechst33342-Tracker Blue were purchased from Beyotime Biotechnology. Cell Counting Kit-8 (CCK-8) was obtained from Topscience (Shanghai, China). The Angeli's Salt were purchased from Nanjing Jiancheng Bioengineering Institute, dissolved into Saturated sodium hydroxide aqueous solution to form aqueous solution and were divided into several parts for daily experiments. To maintain the activity, all these solutions were stored at -80 °C before using. The mice were purchased from Jiangsu Gempharmatech co. ltd. Water was purified and doubly distilled by a Milli-Q system (Millipore, USA). HeLa cells (human cervical cancer cells) and HEK-293T cells (Immortalized cell line of human embryonic kidney cells) were obtained from Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Beijing, China). Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin and 100% heat-inactivated fetal bovine serum were obtained from Thermo Scientific HyClone (MA, USA). The UV-vis absorbance spectra were recorded on a Shimadzu UV-2450 spectrophotometer with an interval of 2 nm. Fluorescence spectra were recorded on an FS5 spectro fluorometer (Edinburgh, UK). Confocal fluorescence imaging was performed on a Nikon A1+ confocal microscope (Japan) with 20× or 60× objective lens. The fluorescent images of mice and serum were obtained via an IVIS Lumina XR Imaging System (Caliper, U.S.A.) equipped with a cooled charge coupled device (CCD) camera. Circular ROIs were drawn over the areas and quantified by Lumina XR Living Image software, version 4.3. Mass spectra were performed using an LCQ Advantage ion trap mass spectrometer (Thermo Finnigan). NMR spectra were recorded on a Bruker DRX-400 spectrometer using TMS as an internal standard. Thin-layer chromatography (TLC) was performed on silica gel aluminum sheets with an F-254 indicator. Column chromatography was conducted using 200-300 mesh SiO_2 (Qingdao Ocean Chemical Products).

1.2 In vitro Assays

Absorption fluorescence and Photoacoustic measurements. Both UV-Vis absorption, fluorescence and photoacoustic measurements were carried out in PBS/DMSO solution (10 mM, pH 7.4, v/v, 4/1). Absorbance spectrum for QL-HNO was recorded in the range from 400 nm to 820 nm. Fluorescence spectrum of QL-HNO was recorded in the range from 740 nm to 850 nm with an excitation wavelength of 720 nm. Photoacoustic spectrum for QL-HNO was recorded in the range from 680 nm to 830 nm. After measuring the emission and absorption spectra of the test solution, the response behavior of QL-HNO to HNO were determined by adding Angeli's salt (AS) into the test solution of QL-HNO. Optoacoustic imaging was conducted in Vision 256-TF Multispectral Optoacoustic Tomographic (MSOT) imaging system (iThera Medical GmbH). Different concentrations of HNO donors (Angeli's salt) was

added to the mixture containing in PBS/DMSO solution (10 mM, pH 7.4, v/v, 4/1) of QL-HNO (10 μM) followed by the photoacoustic properties measurement. The limit of detection was calculated using the following equation:

Detection limit = $3\sigma/k$

Where σ is the standard deviation of the blank measurements, k is the slope between the fluorescence intensity at 761 nm and photoacoustic intensity at 720 nm versus various Angeli's salt concentrations in the linear region.

In order to study the time response of QL-HNO to HNO donors (Angeli's salt), QL-HNO (10 μM) was rapidly mixed with different concentrations of Angeli's salt (20 μM) in PBS/DMSO solution (10 mM, pH 7.4, v/v, 4/1). Then, the fluorescence intensity at 761 nm was recorded at different time (0, 2, 5, 8, 10, 15, 20, 25, 30min) in 30 min at 37 °C with an excitation wavelength of 720 nm on an Edinburgh FLS1000-stm fluorescence spectrophotometer.

For selectivity assay, QL-HNO was first mixed with different biologically relevant species, including KCl (15mM), $ZnCl_2$ (2.5mM), $FeCl_3$ (2.5mM), $MgCl_2$ (2.5mM), $NaNO_3$ (100 μM), Na_2HPO_4 (100 μM), Na_2SO_3 (100 μM), GSH (1mM), Cys (1 mM), Arg (1 mM), Glycine (1 mM), NaS (1 mM), KO_2 (100 μM), H_2O_2 (100 μM), ClO^- (100 μM), especially Angeli's salt (20 μM) in PBS buffers (pH 7.4). Then, the mixture was incubated at 37 °C for 30 min and fluorescence spectra were obtained on an Edinburgh FLS1000-stm fluorescence spectrophotometer. The fluorescence intensity at 761 nm was plotted against different species.

1.3 Cellular Studies

Cell Culture. HeLa cells and HEK-293T were all cultured in DMEM, supplemented with 10% fetal calf serum, 100 U/mL of penicillin, and 100 μg/mL of streptomycin at 37 °C in a humidified atmosphere incubator containing 5 wt %/vol CO_2. The cell density was determined using a TC20™ automated cell counter (BIO-RAD, USA).

Cytotoxicity assay. Cells were seeded in 96-well plates at a density of 1×10^4 cells per well and cultured for 24 h. The cells were incubated with different concentrations of **QL-HNO** (0 - 10 μM) for 1 h. The cells were cultured for 24 h before addition of CCK-8 solution (10 μL). The cells were incubated at 37°C for 2 h and optical density (OD) values at 450 nm were measured with a microplate reader. Cell viability was determined using the following equation:

$$\text{Cells viability (\%)} = (OD_{probe} - OD_{blank}) / (OD_{control} - O_{Dblank}) \times 100$$

where OD_{probe} are the OD values for cells treated with **QL-HNO** (0 μM - 10 μM).

Confocal laser scanning microscopy (CLSM) imaging. HEK-293T and HeLa cells (1×10^6) were inoculated in the 35-mm glass-bottom culture dishes (Φ 35 mm) and incubated for 24 h, the density of cell adhesion on the culture dish was about 50%. For imaging of exogenous HNO in living cells, HEK-293T cells were pretreated with QL-HNO (10 μM) and then incubated with AS (30 μM, 100 μM, and 200 μM, respectively) at 37 ° C for 30 minutes. Then confocal imaging was performed after washing the cells with PBS buffer. For imaging of endogenous HNO in living cells, HeLa cells were pretreated with SNP (1 mM), a commercial NO donor for 10 h, then NaASc (1 mM) and QL-HNO (10 μM) were added. Fluorescence images were obtained after another incubation of 30 min. Then perform HNO imaging after washing the cells with PBS buffer.

Subcellular colocalization assay. For the subcellular colocalization assay, HeLa cells were first pretreated with SNP (1 mM), NaASc (1 mM) and QL-HNO (10 μM) in 1 mL of fresh culture medium. Then, Mito-Tracker Green (100 nM), Lyso-Tracker Green (100 nM) and Hoechst 33342-Tracker Blue (100 nM) were added and incubated for 30 min. To obtain fluorescence signals from Lyso-Tracker Green and Mito-Tracker Green, the fluorescence emission wavelength was collected at 500−540 nm under excitation at 488 nm. To obtain fluorescence signals from Hoechst33342-Tracker Blue, the fluorescence emission wavelength was collected at 450-480 nm under excitation at 405 nm.

Z-axis scan. HeLa cells were first incubated with 10 μM of QL-HNO in 1 mL fresh culture medium for 3 h at 37 °C. Then Mito-Tracker Green (100 nM) was added and incubated for another 30 min. The fluorescence signal for QL-HNO was collected from 690 nm to 780 nm with an excitation wavelength of 640 nm. The fluorescence signal for Mito-Tracker Green was collected from 500 nm to 540 nm with an excitation wavelength of 488 nm. The Z-axis scan range was set from -5 μm to 5 μm.

1.4 In vivo Studies

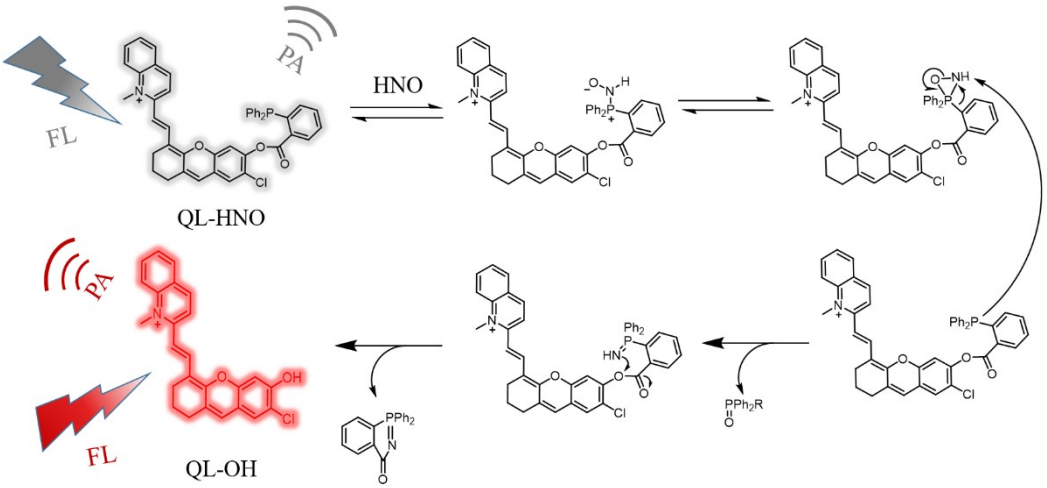
Tumor Mouse Model. All mice experiments were approved by the Hunan Provincial Science & Technology Department and performed in compliance with the guidelines of the Institutional Animal Care and Use Committee of Hunan University (approval number: SYXK2018-0006). Female BALB/c nude mice (4 weeks old, 18-22 g) were used for animal tumor models. A suspension of 1×10^6 HeLa cells was implanted into the right side of each mouse's hip. Tumors were grown to ~100 mm^3 before imaging.

Preparation of In Vivo PA/NIRF Imaging. In vivo NIRF and PA imaging were conducted in accordance with the same following steps. The nude mice bearing HeLa tumors were separated into three groups and pretreated with different conditions. Each group included five mice. The first group was injected with sterilized PBS (50 μL) as the control group. The second group was injected into a QL-HNO probe (100 μM in 50 μL of sterilized PBS) as the sample group. The third group was preinjected with SNP

(1 mM) solutions in 50 μL of sterilized PBS) for 10 h before injection into the NaASc (1 mM) and QL-HNO probe (100 μM in 50 μL of sterilized PBS). In addition, in vivo NIRF and PA images were also acquired before injection (0 min) and at different time points (0, 15, 30, 60, 90, 120, 240, 720 min) after intratumoral injection with the QL-HNO probe (100 μM, 50μL). All of the mice were anesthetized with 1% isoflurane in oxygen before PA/NIRF imaging.

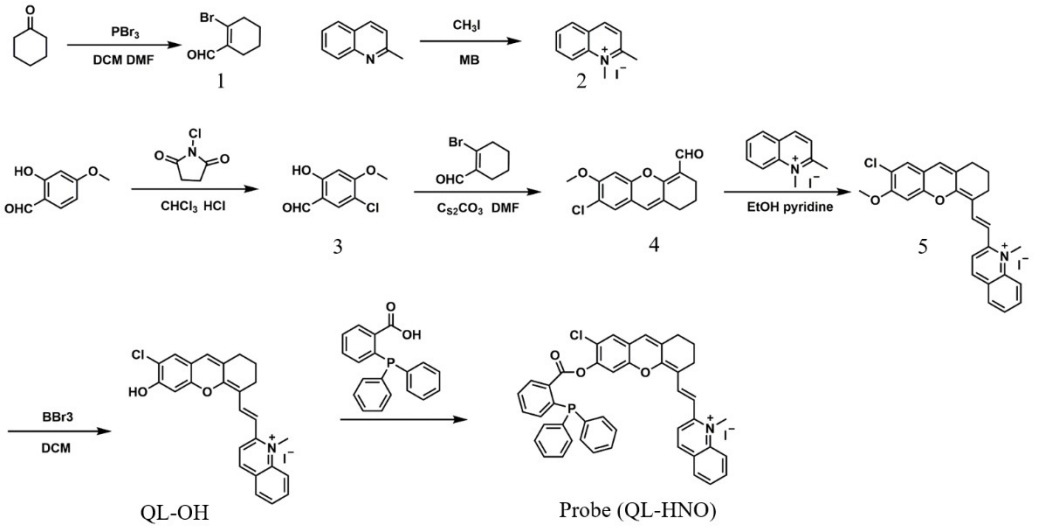
In Vivo PA/NIRF Imaging. For in vivo NIRF imaging, all fluorescence images of mice were captured on an IVIS Lumina XR small-animal imaging system (Caliper, Switzerland) with a Cy5.5 filter (690−760 nm). The anesthetized mice were positioned in the imaging cabin with the tumors placed in the field of view. All imaging parameters were kept constant throughout the experiments. Data were analyzed with Living Image 4.0 Software. ROIs of equal areas in the tumor regions were obtained for further analysis. For in vivo PA imaging, all PA images of mice were captured on a multispectral optoacoustic tomographic (MSOT) imaging system (inVision256-TF, iThera Medical GmbH). Each mouse was fixed with a double leg slide and placed in a 32 °C water bath with the excitation at 680 nm through 850 nm with an interval of 10 nm. The entire tumor was scanned with each wavelength with a spacing of 0.3 mm. Guided ICA spectral unmixing was introduced to separate signals from the activated probes and those from photoabsorbers in tissues (e.g. hemoglobin). The mean PA intensities were obtained with ViewMSOT from five different ROIs from the tumor.

Metabolization of QL-HNO. The metabolization of QL-HNO in mice was conducted with an IVIS animal imaging system. QL-HNO (100 μM, 50 μL) was injected to mice bearing HeLa tumors and mice were euthanized for 12 h. The major organs, such as heart, liver, spleen, lung, and kidney, and tumor were carefully harvested and placed onto petri dishes after washing with saline. HeLa tumor bearing mice with injection of PBS was used as a control. Fluorescence images of mice were captured on an IVIS Lumina XR small animal imaging system (Caliper, Switzerland) with the Cy5.5 filter (690-760 nm). The fluorescence intensity of each organ and tumor was analyzed using the Living Image 4.0 Software.



Scheme S1. Proposed mechanism for the reaction of QL-HNO with HNO.

1.5 Synthesis of compounds



Scheme S2. Synthesis of probe QL-HNO

Synthesis of 2-bromocyclohex-1-ene-1-carbaldehyde (Compound 1): To DMF (5 mL, 65 mmol) and $CHCl_3$ (25 mL) at 0 °C was slowly added PBr_3 (6 mL, 64 mmol). After 45 min, cyclohexanone (5 mL, 48.4 mmol) was added and the mixture was stirred for 12 h at room temperature. The resulting Orange solution was then poured into ice water. The layers were separated and the aqueous layer was extracted with CH_2Cl_2 (100 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated *in vacuo* to provide a yellow oil with a yield of 53%, which was pure enough to be used directly in the next step.

Synthesis of 1,2-dimethylquinolin (Compound 2): To a solution of 2-Methylquinoline (2 g, 14.0 mmol) in acetonitrile or toluene were added methyl iodide (2 g, 14.2 mmol) and the reaction mixture was heated at 85 °C reflux for 12 h. The resulting solid was filtered under vacuum, washed with Ethyl Acetate and dried to afford Compound 2 with a yield of 82%.

Synthesis of 5-Chloro-2-hydroxy-4-methoxybenzaldehyde (Compound 3): To a mixture of 2-hydroxy-4-methoxybenzaldehyde (5.2 g, 34.2 mmol) and N-Chlorosuccinimide (5.8 g 43.6 mmol) in chloroform (100 mL) was added concentrated HCl (2 mL) dropwise and then the mixture was heated to reflux under N_2 for 4 h. The cooled mixture was washed with water (3×100 mL) and 10% sat $NaHCO_3$ (100 mL), and then it was dried (Na_2SO_4) and filtered and concentrated in vacuo. The crude product was purified by silica gel column chromatography with PE/AcOEt (6/1, v/v) as the eluent to give Compound 3 as a light beige solid with a yield of 88.9%. ^1H NMR (400 MHz, $CDCl_3$) δ (ppm): 11.44 (1H, s, OH), 9.71 (1H, s, CHO), 7.52 (1H, s, Ar-H),

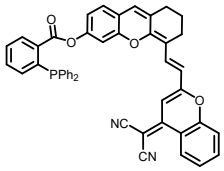
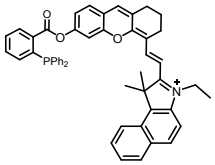
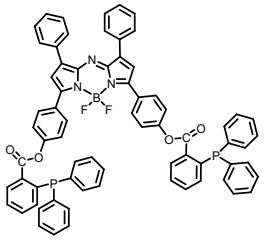
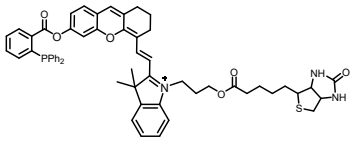
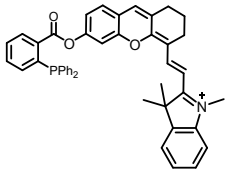
6.51 (1H, s, Ar-H), 3.97 (3H, s, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 193.59, 163.38, 161.77, 134.23, 114.71, 114.08, 100.31, 56.55.

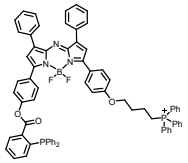
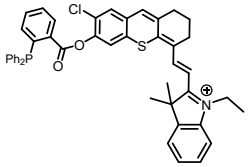
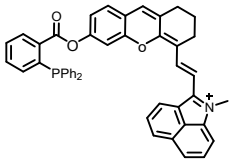
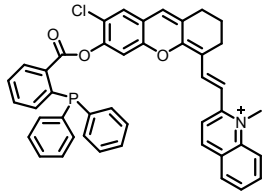
Synthesis of 7-chloro-6-methoxy-2,3-dihydro-1H-xanthene-4-carbaldehyde (Compound 4): To 2- bromocyclohex-1-ene-1-carbaldehyde **Compound 1** (150 mg, 0.8 mmol) in DMF (4 mL) at 25 °C were **Compound 3** (140 mg, 0.75 mmol) and Cs_2CO_3 (735 mg, 2.25 mmol). The medium was stirred for 12 h at 35 °C and an intense yellow spot appeared on the TLC plate. The solution was then filtered on a filter paper and the filtrate was concentrated. The resulting residue was dissolved in CH_2Cl_2 (30 mL) and washed with H_2O (2 × 30 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography with PE/AcOEt (6/1, v/v) as the eluent to give Compound 4 as a yellow solid with a yield of 79.7%. ^1H NMR (400 MHz, $CDCl_3$) δ (ppm): 10.31 (1H, s, CHO), 7.16 (1H, s, Ar-H), 6.70 (1H, s, Ar-H), 6.56 (1H, s, =CH), 3.94 (3H, s, CH_3), 2.57 (2H, t, J = 6.0 Hz, CH_2), 2.45 (2H, t, J = 6.0 Hz, CH_2), 1.69-1.75 (2H, m, CH_2). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 187.40, 160.30, 156.22, 152.08, 127.99, 127.17, 125.53, 117.42, 115.02, 113.22, 99.87, 56.65, 29.95, 21.51, 20.36.

Synthesis of (E)-2-(2-(7-chloro-6-methoxy-2,3-dihydro-1H-xanthen-4-yl)vinyl)-1-methylquinolin-1-ium iodide (Compound 5)： To a solution of Compound 4(80 mg, 0.31 mmol) in contain catalytic amount of piperidine anhydrous EtOH were added 1, 2-dimethylquinolin (Compound 2) (132 mg 0.46 mmol). After 12 h at 80 °C the deep blue solution obtained. The solvent was removed by rotary evaporation, and the resulting residue was purified by column chromatography using DCM/MeOH (20/1, v/v) as the eluent to yield Compound 5 as a blue solid with a yield of 42%. ^1H NMR (400 MHz, CD_3OD) δ (ppm): 8.98 (1H, d, J = 6.8 Hz, Ar-H), 8.64 (1H, d, J = 8.4 Hz, Ar-H), 8.36 (1H, d, J = 8.8 Hz, Ar-H), 8.15-8.29 (2H, m, Ar-H), 8.08 (1H, d, J = 15.2 Hz, =CH), 7.93 (1H, t, J = 7.6 Hz, Ar-H), 7.49 (1H, d, J = 14.8 Hz, Ar-H), 7.16 (2H, d, J = 7.6 Hz, =CH), 6.43 (1H, s, Ar-H), 3.34 (3H, s, CH_3), 2.73 (2H, t, J = 6.0 Hz, CH_2), 2.64 (2H, t, J = 6.4 Hz, CH_2), 2.36 (3H, s, CH_3), 1.90-1.96 (2H, m, CH_2). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 168.48, 153.46, 146.38, 145.60, 138.63, 138.04, 137.76, 135.01, 131.61, 129.41, 128.05, 127.96, 126.90, 126.64, 125.99, 124.50, 119.62, 118.63, 117.57, 114.85, 54.60, 42.41, 29.38, 20.55, 18.95. MS (ESI) m/z: $[M-I]^+$ calcd 416.14, found 416.26.

Synthesis of QL-OH： BBr_3 (0.45 mL, 4.5 mmol) was added to a solution of Compound 5 (122 mg 0.22 mmol) in dry CH_2Cl_2 (6 mL) at 0 °C and the solution was stirred at room temperature for 16 h. Then saturated $NaHCO_3$ solution (30 mL) was added to quench the reaction, followed by extraction of the aqueous layer with CH_2Cl_2. The organic layer was dried over Na_2SO_4 and the solvent was removed by evaporation under reduced pressure, and the obtained crude QL-OH was directly used in the following reaction without further purification. MS (ESI) m/z: $[M-I]^+$ calcd 402.13, found 402.75.

Table S1 Representative imaging probe for nitroxyl.

Probe and Reference	λex/em (nm)	Imaging mode	Response Time	Detection Limit	Biological Application
 Talanta 193 (2019) 152-160.	580/740	NIRF	30 min	50 nM	Living cells
 Anal. Chem. 93 (2021) 6551–6558	695/725	NIRF	15 min	160 nM	Living cells; Mouse drug-induced liver injury model
 Analyst 140 (2015) 4576	682/734	NIRF	20 min	30 nM	Living cells
 Chem. Commun. 57 (2021) 5063	670/708	NIRF	30 min	57 nM	Living cells; Tumor-bearing mice model
 J. Mater. Chem. B 4 (2016) 1263	670/710	NIRF	45 min	622 nM	Living cells; Live mouse intraperitoneal injection

 J. Mater. Chem. B 7 (2019) 305	680/730	NIRF	30 min	50 nM	Living cells; Live mouse intraperitoneal injection
 Sens. Actuators B Chem 384 (2023) 133646.	λex: 630 λem:-720/783	NIRF/PA	30 min	110 nM	Ratiometric NIRF/PA dual-modal imaging in living cells and animals
 Chem. Commun. 59 (2023) 8969	PA:680/795	PA	20 min		Live mouse prodrug release and liver injury
 This work	720/761	NIRF/PA	30 min	NIRF: 18.0 nM PA: 27.1 nM	Living cells; NIRF/PA dual-modal imaging in tumor-bearing mice model

2. NMR and MS spectra

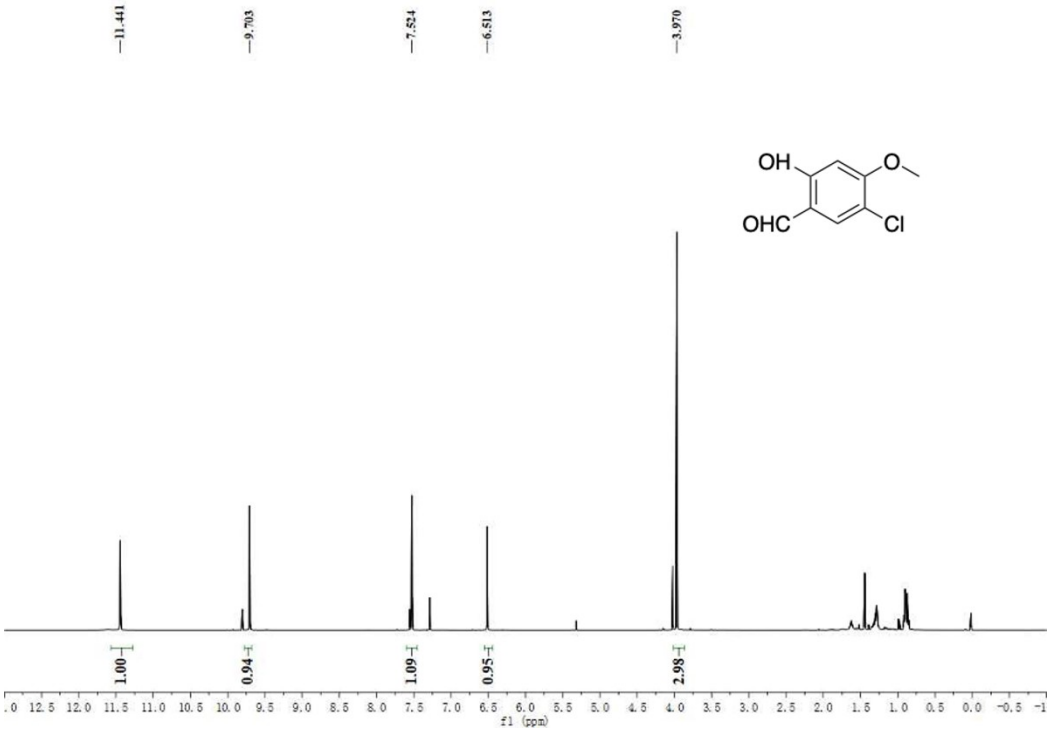


Fig. S1 ^{1}H NMR spectrum of Compound 3 in $CDCl_3$.

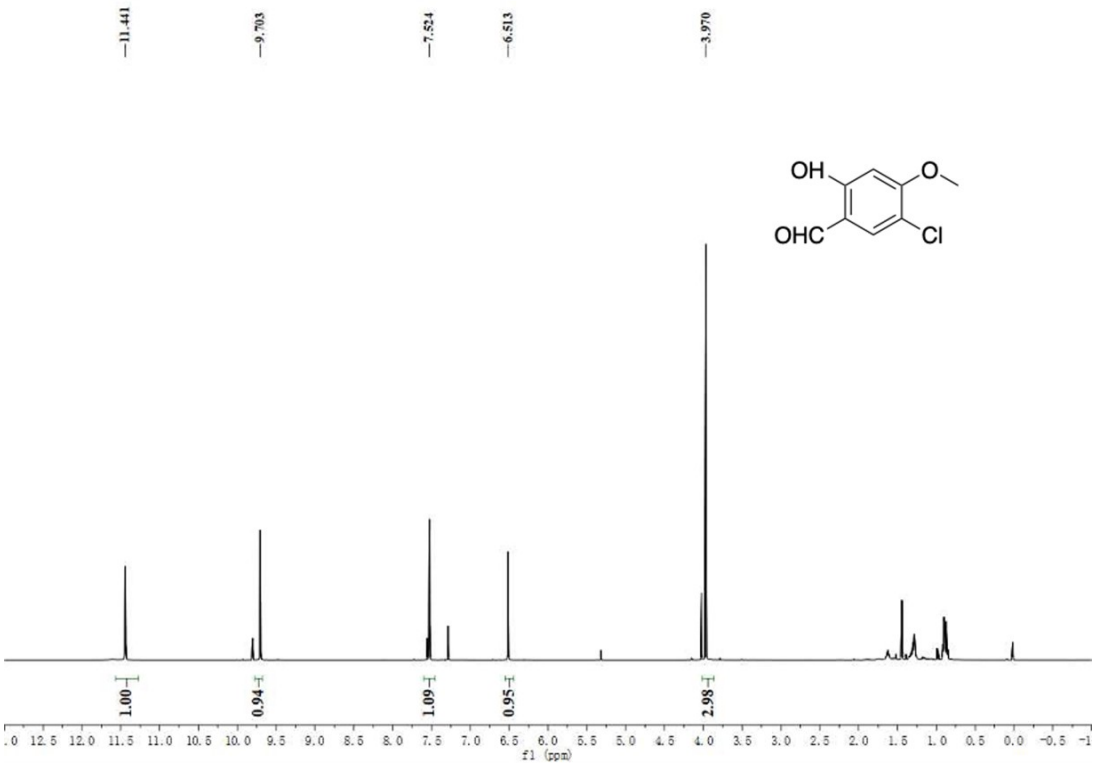


Fig. S2 ^{13}C NMR spectrum of Compound 3 in $CDCl_3$.

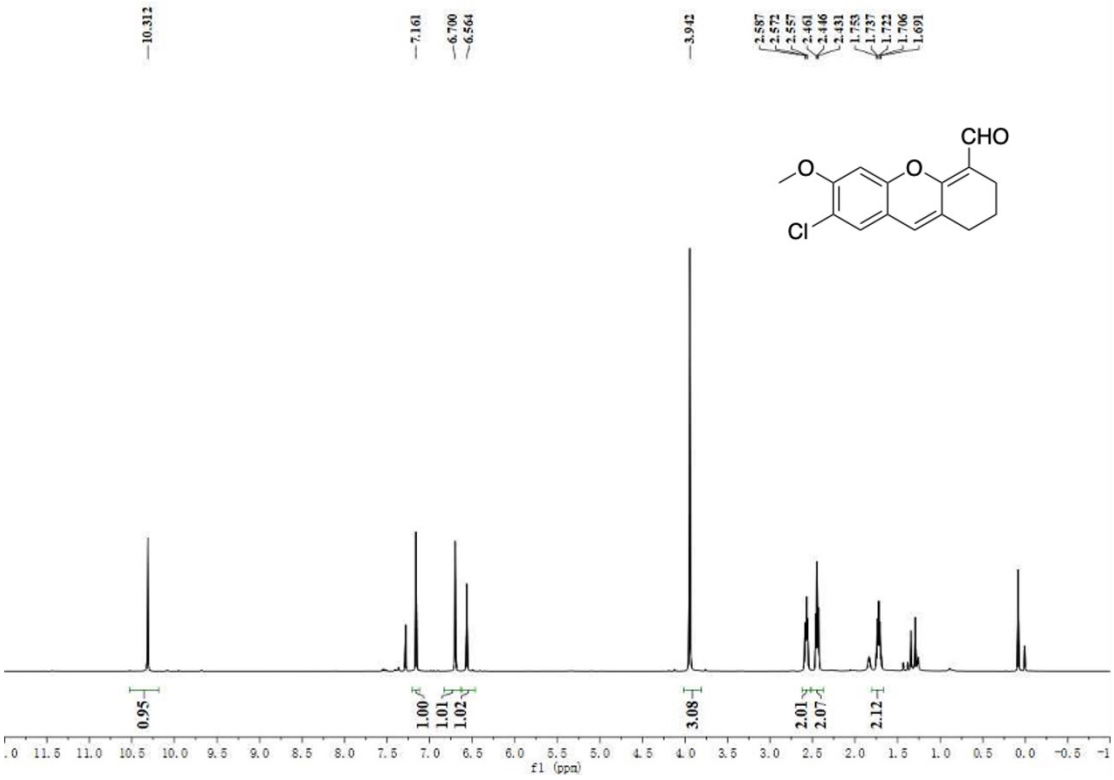


Fig. S3 ^{1}H NMR spectrum of Compound 4 in $CDCl_3$.

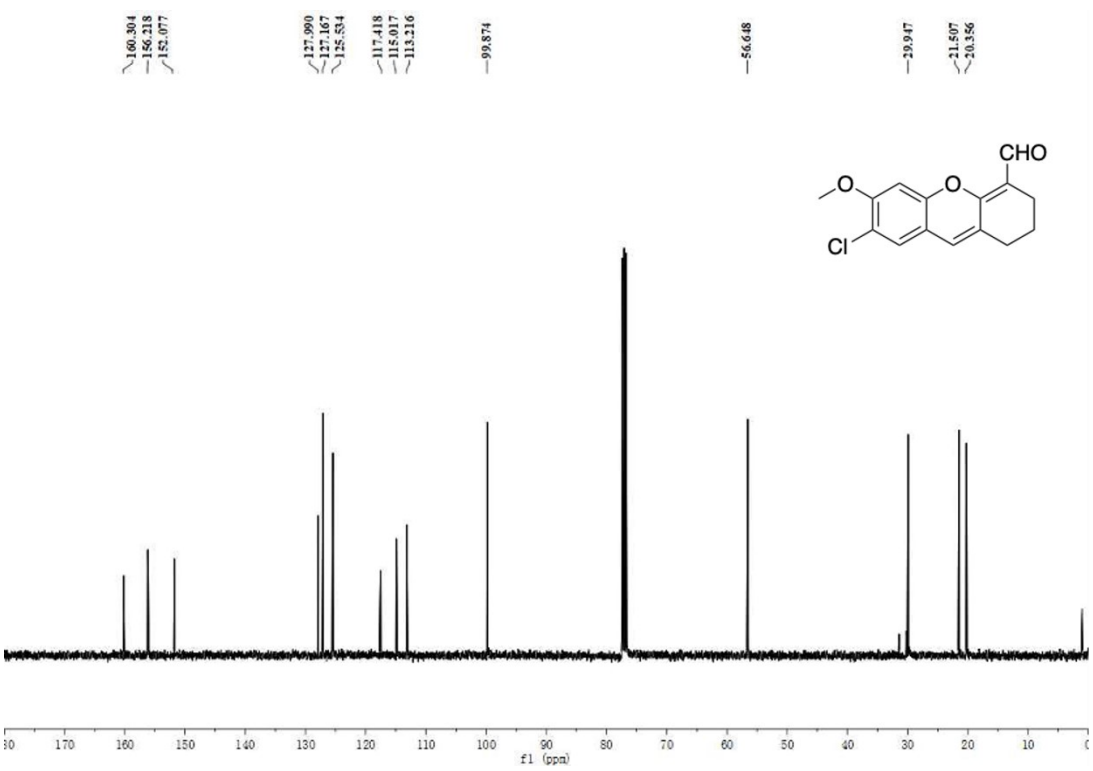


Fig. S4 ^{13}C NMR spectrum of Compound 4 in $CDCl_3$.

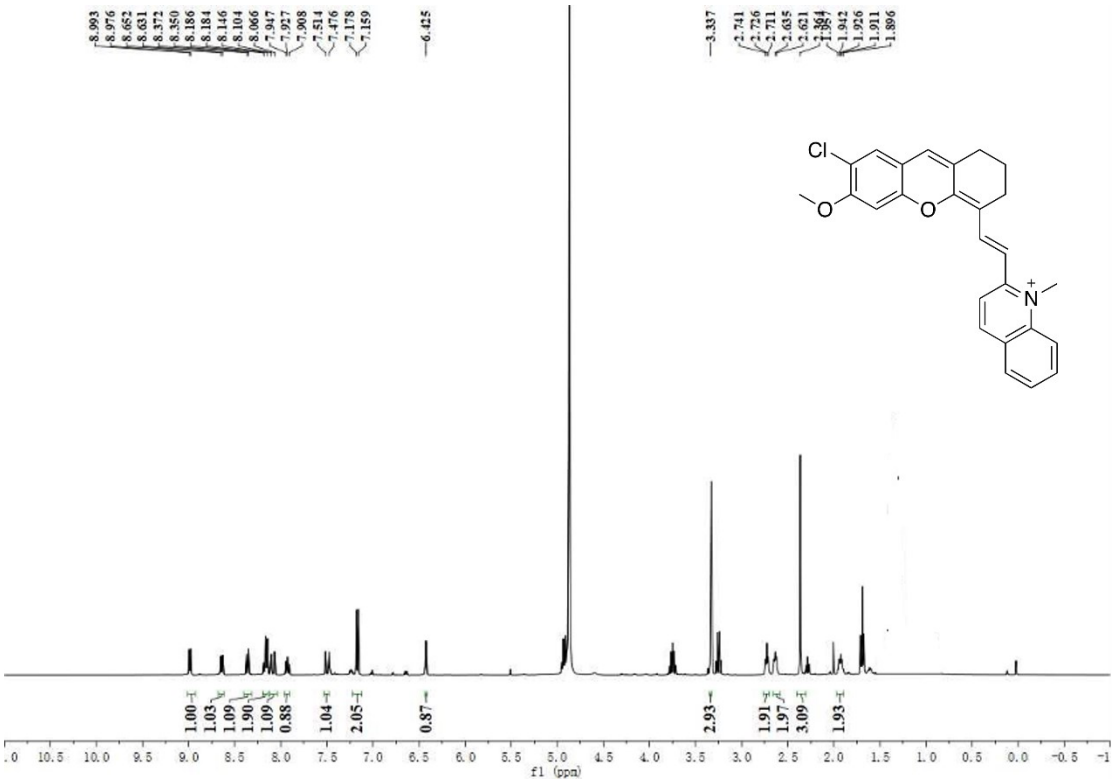


Fig. S5 ^{1}H NMR spectrum of Compound 5 in CD_3OD.

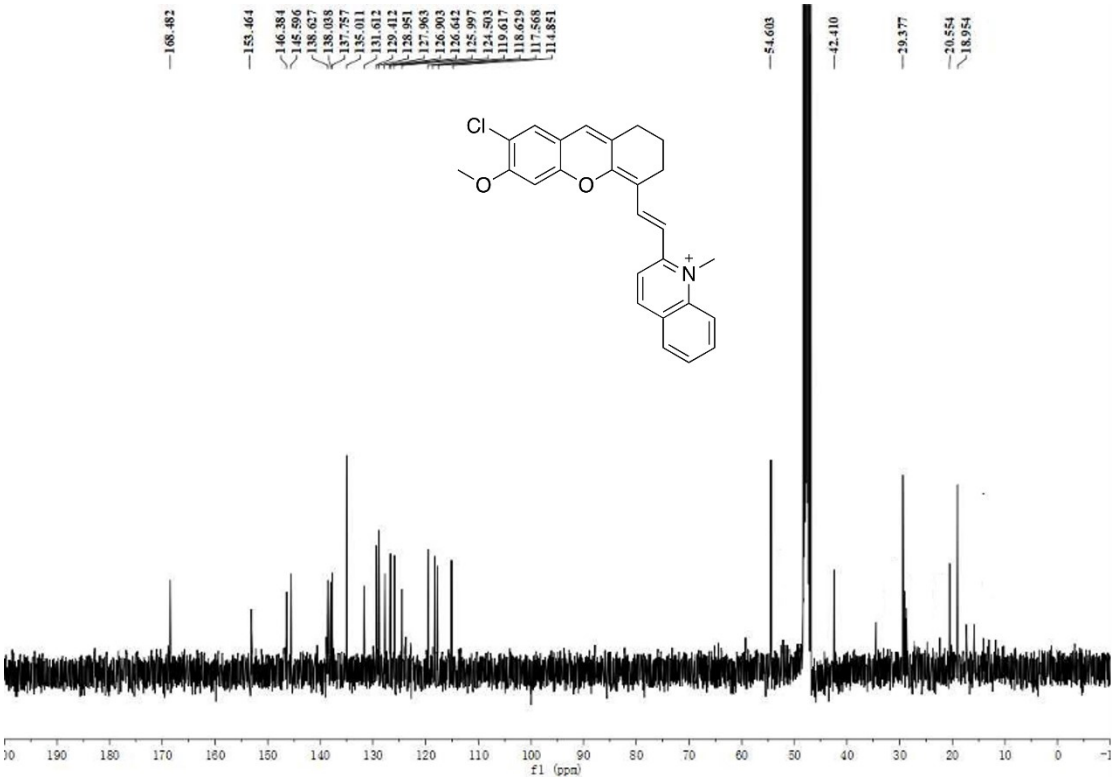


Fig. S6 ^{13}C NMR spectrum of Compound 5 in CD_3OD.

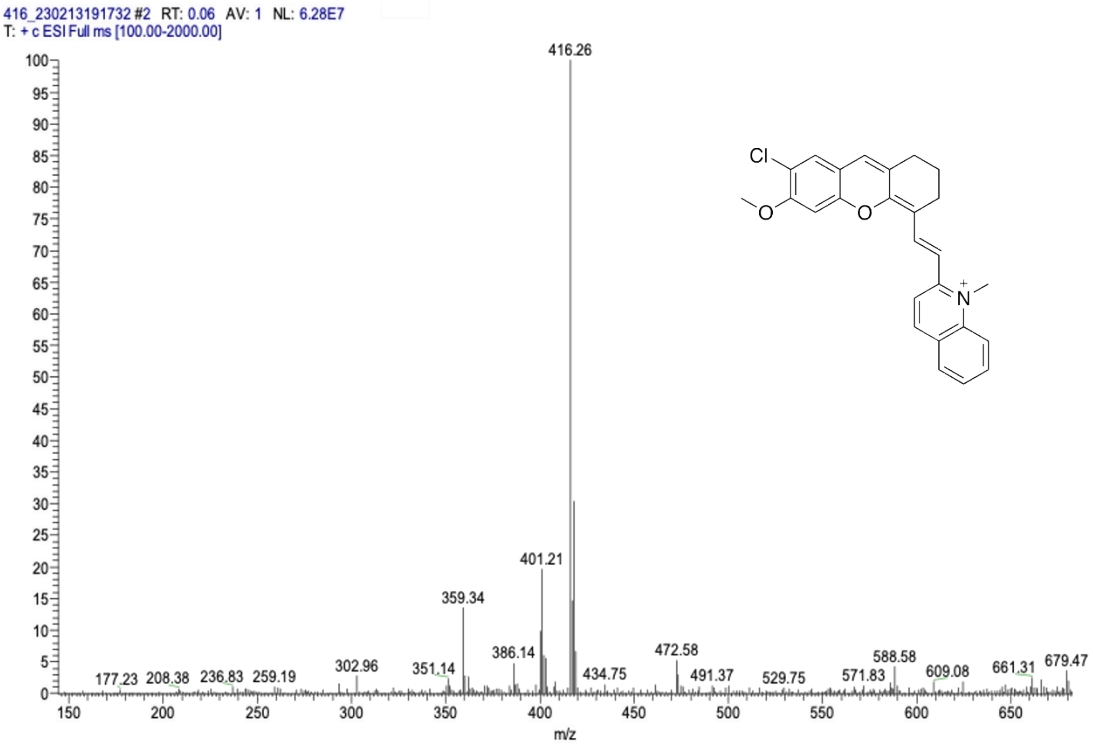


Fig. S7 ESI-MS spectrum of Compound 5.

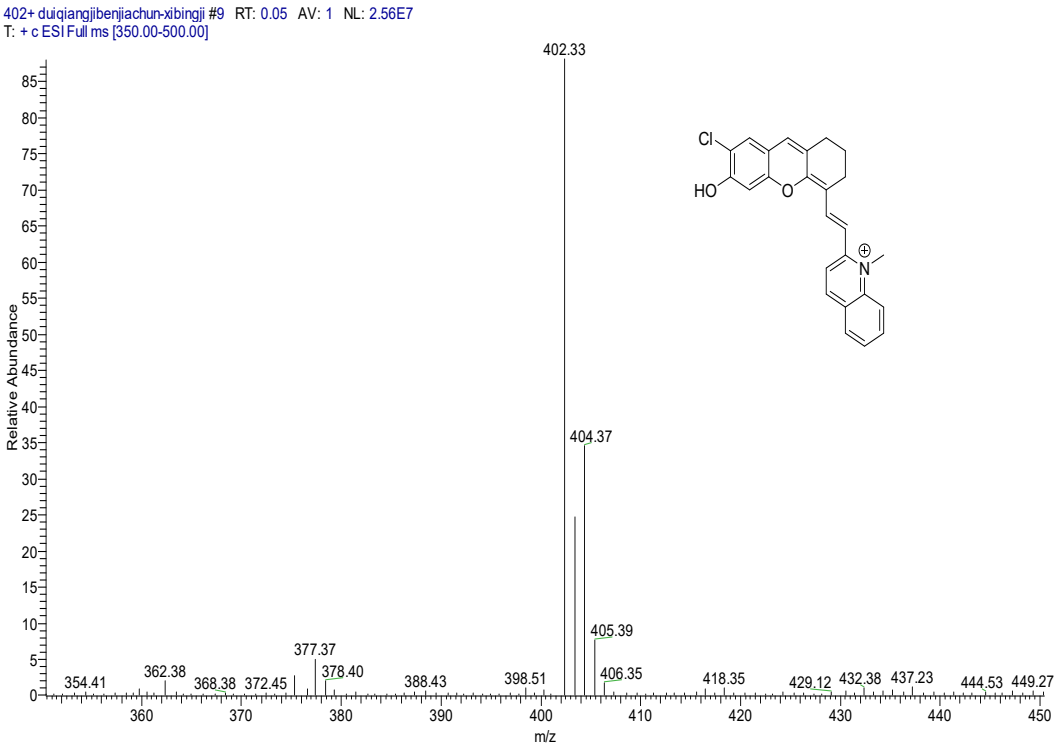


Fig. S8 ESI-MS spectrum of the QL-OH

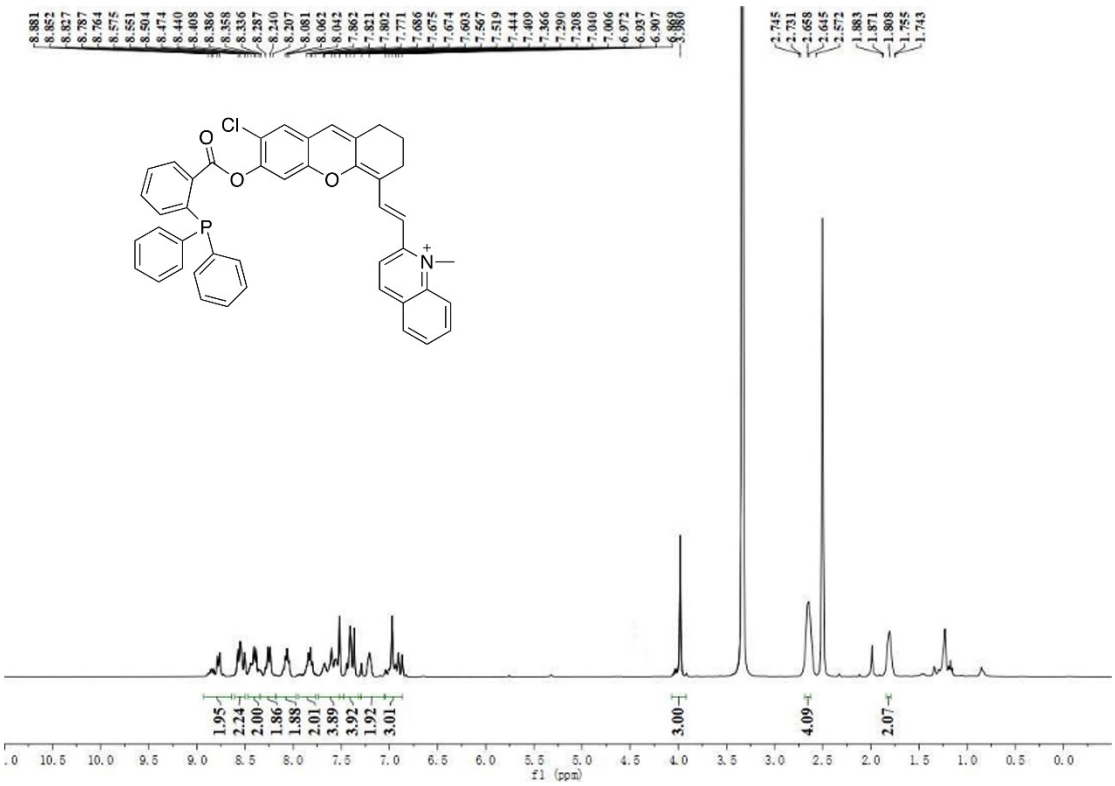


Fig. S9 ^{1}H NMR spectrum of QL-HNO in DMSO-*d*.

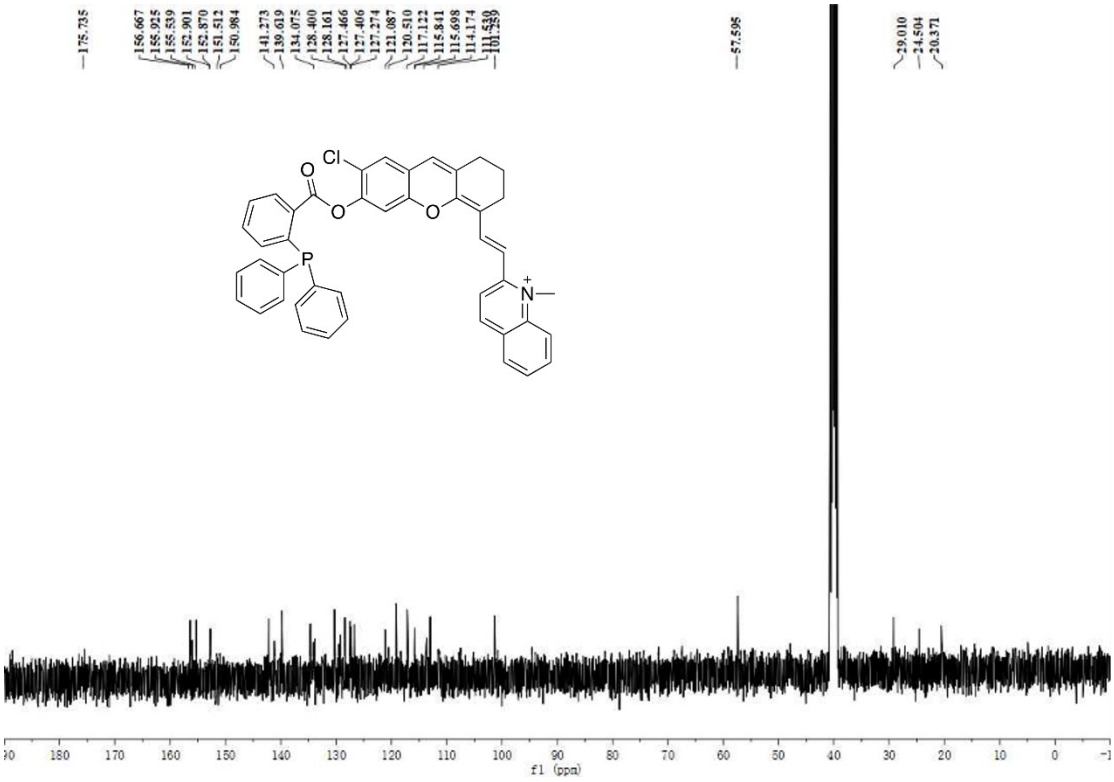


Fig. S10 ^{13}C NMR spectrum of QL-HNO in DMSO-d_6.

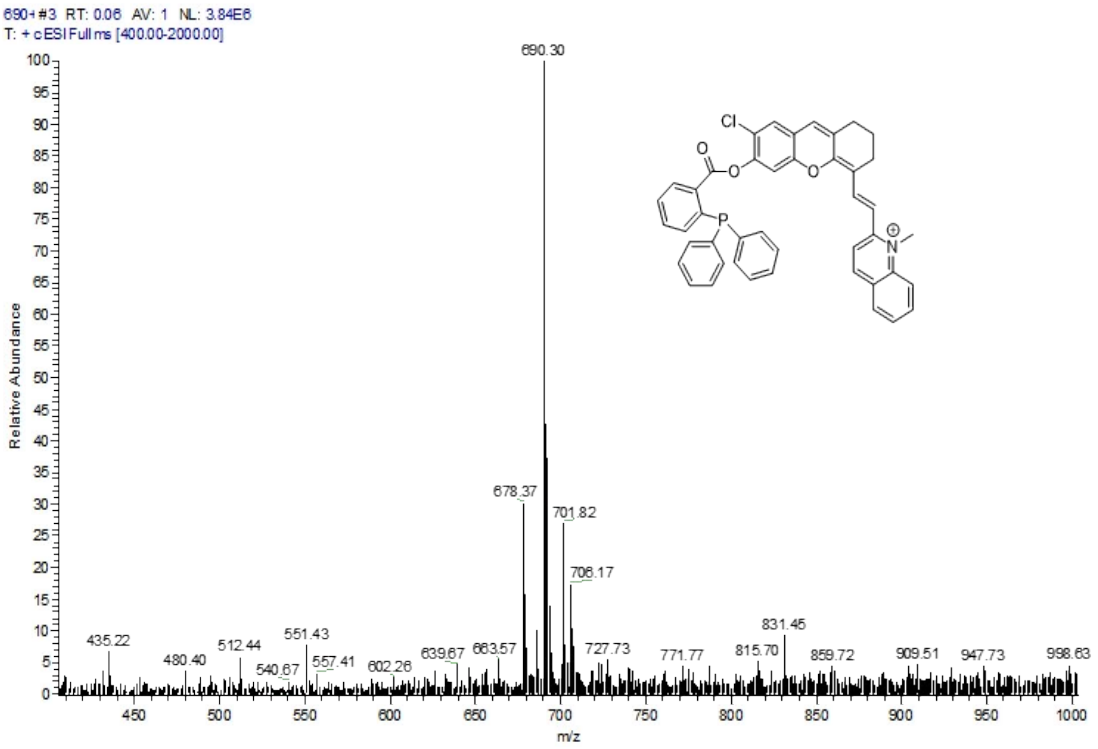


Fig. S11 ESI-MS spectrum of QL-HNO.

3. Additional Figures

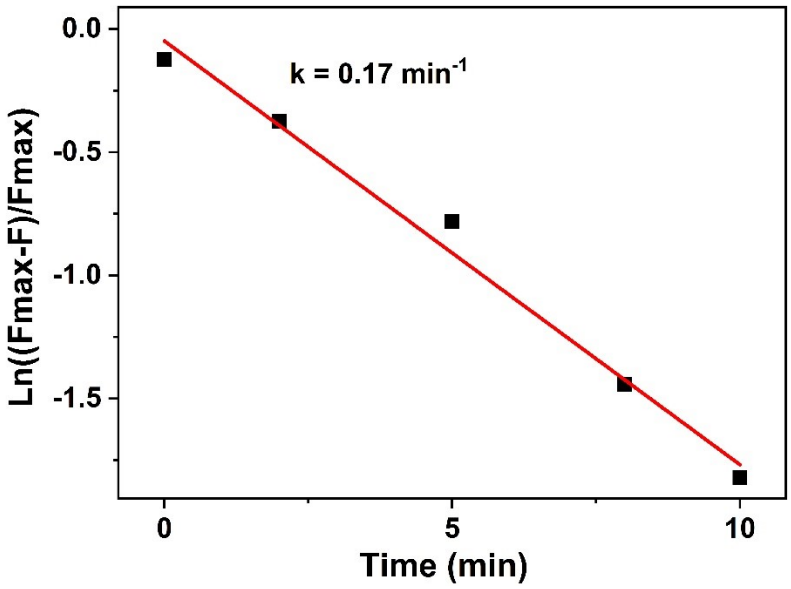


Fig. S12 Pseudo ffrst-order kinetic plot of the reaction of QL-HNO (10 μM) with HNO (20 μM).

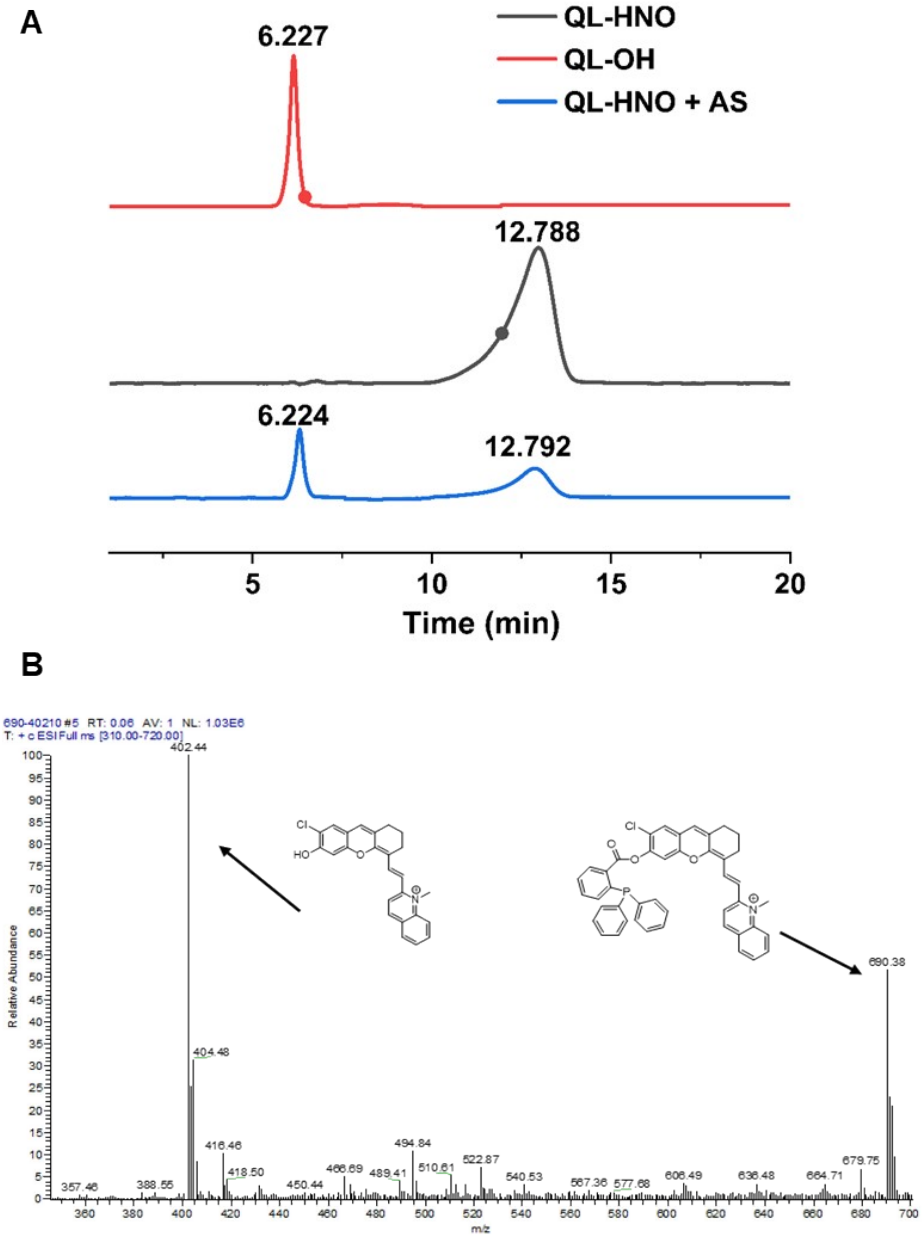


Fig. S13 (A) Chromatograms of different reaction systems: QL-OH; QL-HNO; QL-HNO reacting with Angeli's salt (from top to bottom). (B) ESI-MS spectrum of the reaction between QL-HNO and Angeli's Salt.

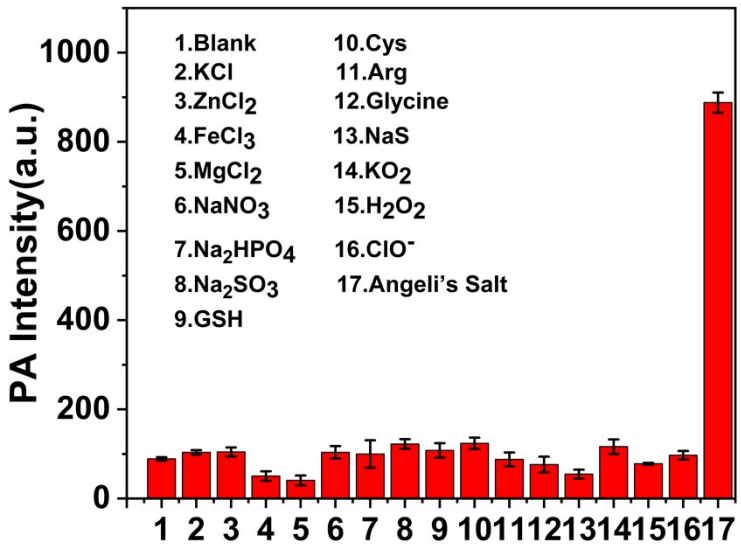


Fig S14. Photoacoustic of the QL-HNO solution toward different relevant substances at 37 °C for 30 min. The error bars represent the standard deviation from three separate measurements.

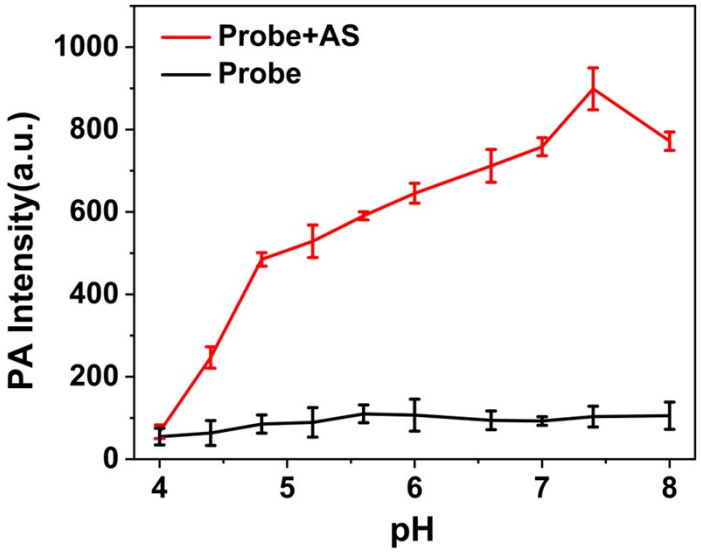


Fig S15. The Photoacoustic effects of pH on the catalytic activity of HNO. QL-HNO (10 μM) and QL-HNO (10 μM) reacted with Angeli's Salt (20 μM) in different pH buffer for 30 min at 37 ℃. The error bars represent the standard deviation from three separate measurements.

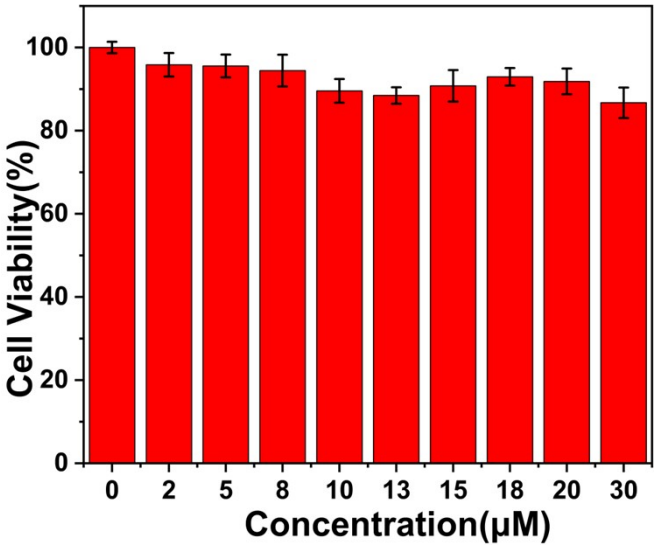


Fig S16. Dark cytotoxicity effect of QL-HNO (0 – 30 μM) on HeLa cells.

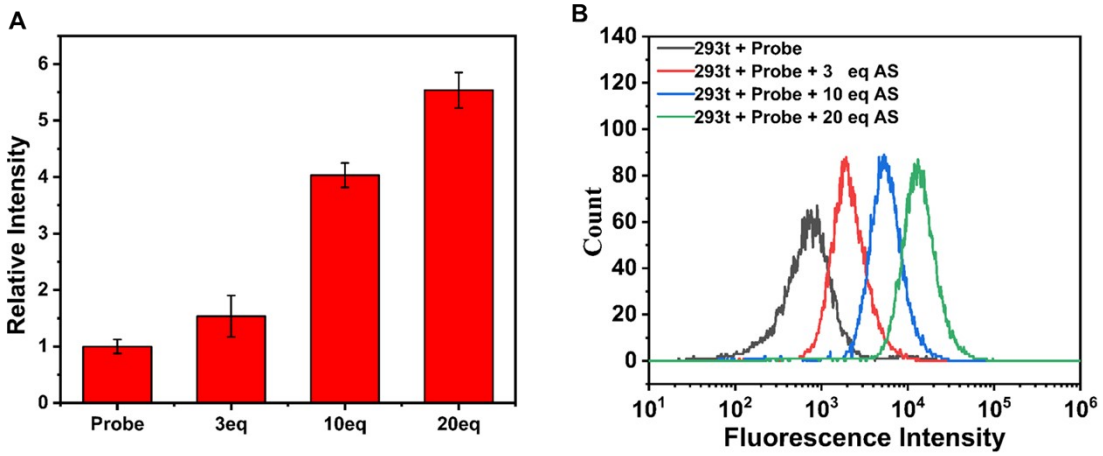


Fig S17. (A) Relative fluorescence intensity of HEK-293T cells cultured with 10 μM QL-HNO under different HNO donor. (B) The flow cytometric analysis of HEK-293T cells under different concentrations of HNO donor for 30 min.

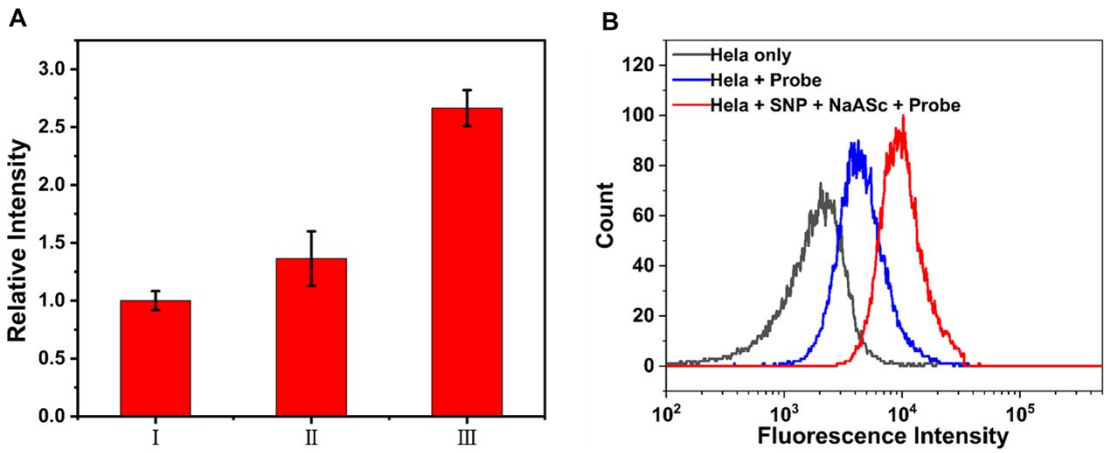


Fig S18. (A) Relative fluorescence intensity of HeLa cells cultured with 10 μM QL-HNO under different condition, from left to right: control group, cells incubated with10 μM QL-HNO, cells treated with SNP (1 mM) for 10 h followed by NaASc (1 mM) and QL-HNO (10 μM). (B) The flow cytometric analysis of in HeLa cells under different condition.

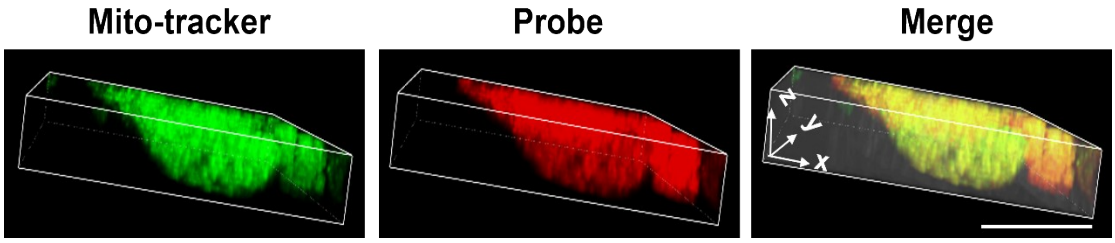


Figure S19. Fluorescence imaging through the z-axis of HeLa cells incubated with QL-HNO (10 μM) for 30 min and followed by 100 nM Mito-Tracker Green for 30 min. Scale bar = 10 μm.

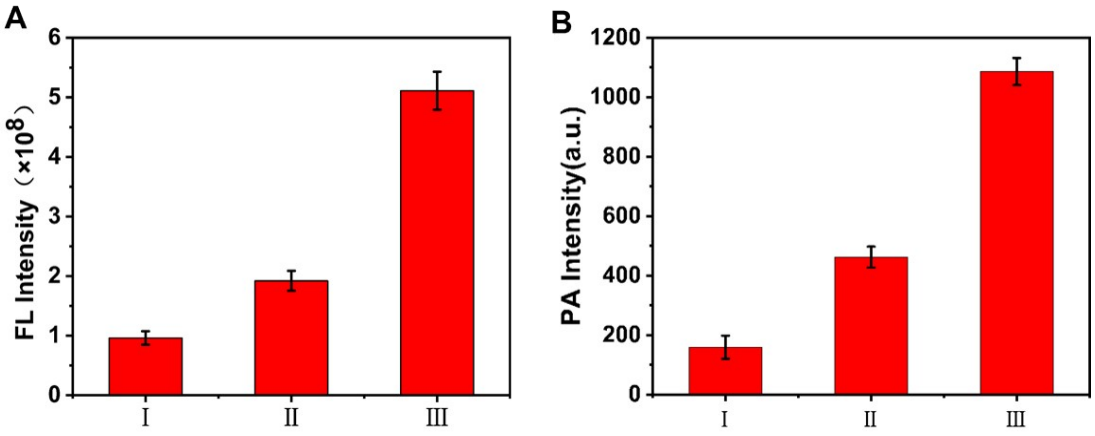


Fig S20. (A) Corresponding NIRF intensity of images of nude mice (n = 3) with 10 μM QL-HNO under different condition, from left to right: control group, cells incubated with10 μM QL-HNO, cells treated with SNP (1 mM) for 10 h followed by NaASc (1 mM) and QL-HNO (10 μM). (B) Corresponding PA intensity of images of mice (n = 3) treated under the conditions as noted in panel Fig. S19A.

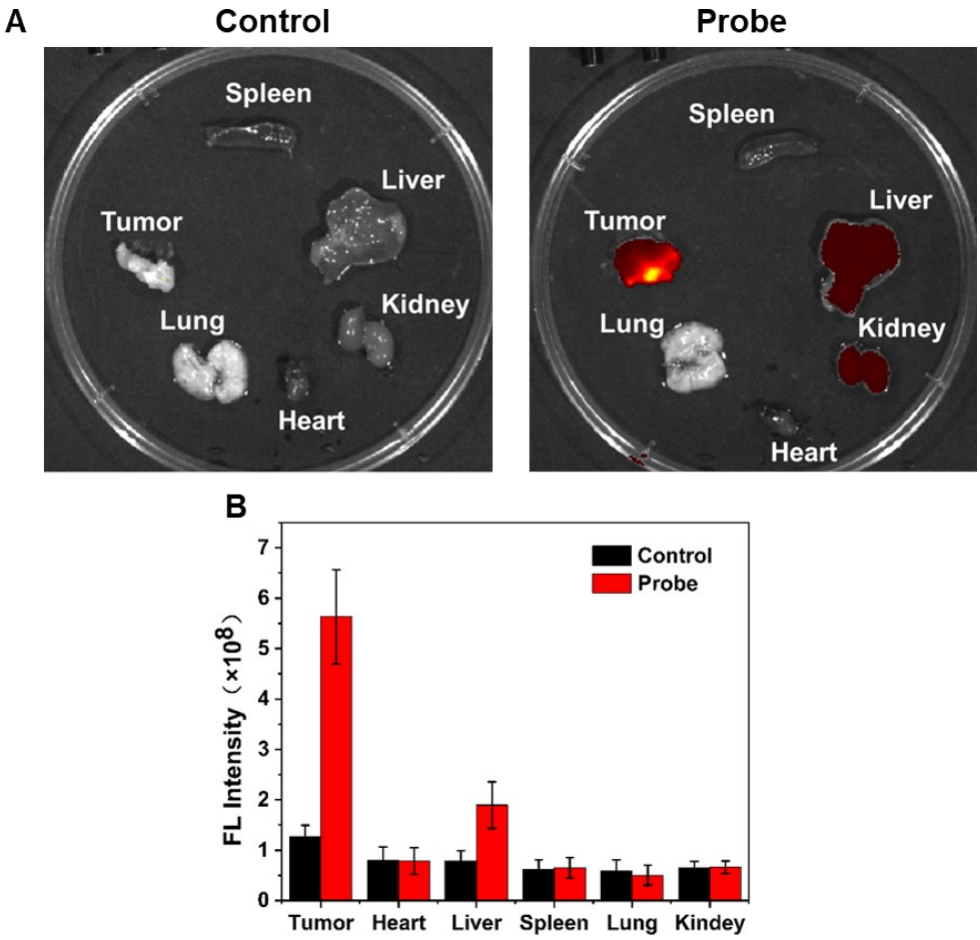


Fig S21. (A) Ex-vivo fluorescence images of major organs (heart, liver, spleen, lung, and kidney) and tumors dissected at 12 h after intratumoral injection of QL-HNO from the mice bearing HeLa tumor; (B) Representative fluorescence intensity of major organs and tumors in HeLa tumor bearing mice at 12 h after intratumoral injection of QL-HNO as noted in (A). The error bars represent the standard deviation from three separate measurements.