Supporting Information

Engineering a near-infrared LAP fluorescent probe with high sensitivity and selectivity for surgical resection of liver cancer

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1. Materials and General Experimental Methods

Unless otherwise stated, all reagents were purchased from commercial suppliers and used without further purification. Solvents used were purified by standard methods prior to use. Twice-distilled water was used throughout all experiments. Mass spectra were performed using an LCQ advantage ion trap mass spectrometer from Thermo Finnigan. NMR spectra were recorded on a Bruker-500 spectrometer. Photoluminescent spectra were recorded at room temperature with a HITACHI F7100 fluorescence spectrophotometer (1 cm standard quartz cell). A Techcomp UV2600 spectrophometer (China) was used to record the absorbance spectrum. TLC analysis was performed on silica gel plates and column chromatography was conducted over silica gel (mesh 200-300), both of which were obtained from the Yantai Jiangyou Silica Gel Development Company Limited.

2. Determination of the Detection Limit

The detection limit (LOD) was obtained by $3\sigma/k$,[1] where σ is the standard deviation of the blank measurements three times, and k is the slope of the fluorescence intensity versus the concentration of LAP.

3. Spectrometric Studies

Measurement of photophysical properties. For photophysical characterization, the compound **NOH** and probe **NLAP** were dissolved in DMSO to make the stock solutions (500 μM), which were diluted to 5 μM as the testing solutions with PBS buffer solution (25 mM, 10% DMSO, pH 7.4). Absorption and fluorescence spectroscopic studies were performed on a Techcomp UV2600 spectrophometer; a Hitachi F-7100 fluorescence spectrophotometer.

4. Fluorescence Microscopic Studies

Cell culture. L02, HeLa and HepG2 cells were cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM, Hyclone) supplemented with 10% fetal bovine serum (FBS, BI), and 1% antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin, Hyclone) at 37 °C and 5% CO_2. Cells were carefully harvested and split when they reached 80% confluence to maintain exponential growth.

Cell cytotoxicity in MTT assay. Cells were plated in 96-well flat-bottomed plates at 1×10^5 cells per well and allowed to grow overnight prior to exposure to **NLAP**. Then the MTT (0.5 mg/mL) reagent was added for 4 hours at 37 °C and DMSO (100 μL/well) was further incubated with cells to dissolve the precipitated formazan violet crystals at 37 °C for 15 min. The absorbance was measured at 490 nm by a multidetection microplate reader. The following formula was used to calculate the viability of cell growth: Cell viability = mean of A value of treatment group / mean of A value of control.

Fluorescence microscopic imaging. Microscopic imaging uses Zeiss-LSM880 confocal microscope with an excitation filter of 633 nm and a collection wavelength range of 658-747 nm. To avoid the artifacts that occur during fixation procedures, all experiments were conducted in live cells.

For endogenous LAP imaging experiments in live HepG2 cells can be divided into four groups. The first group is that HepG2 cells were incubated with 5 μM **NLAP** for 30 min. The cells were

then washed with PBS buffer before imaging. In the second-fourth group, HepG2 cells were pretreated with bestatin (50-200 μM) for 60 min, respectively, then washed, and incubated with 5 μM **NLAP** for 30 min prior to imaging.

For endogenous LAP imaging experiments in different cells line (L02, HeLa and HepG2), these cells were incubated with 5 μM **NLAP** for 30 min. The cells were then washed with PBS buffer before imaging.

For CP/Bestatin-stimulated cell imaging using **NLAP**, HepG2 cells was divided into three groups. The first group is to incubate cells with **NLAP** (5 μM, 30 min). In the second group, cells were pretreated with CP (2 mg/L) for 12 h, then washed and incubated with **NLAP** (5 μM, 30 min) prior to imaging. In the third groups, cells were incubated with bestatin (200 μM, 1 h) after CP treatment. Then the cells were treated with **NLAP** (5 μM, 30 min) prior to imaging.

5. Determining the subcellular location of NLAP

Live HepG2 cells were cultured in 35-mm glass bottom culture dishes. Cells were first incubated with **NLAP** (5 μM) for 30 min at 37 °C. Then, Mito-Tracker Green (1.0 μM) and Lyso-Tracker Green (1.0 μM) was added and incubated for another 15 min. Cells were washed with PBS prior to imaging. Mito-Tracker Green/Lyso-Tracker Green: λ_{ex} = 488 nm, λ_{em} = 493-620 nm); **NLAP**: λ_{ex} = 633 nm, λ_{em} = 658-747 nm.

6. Visualization of LAP activity in tumor-bearing mice

All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Nanhua University. For establishing a mouse tumor model, the HepG2 cells (1×10^6 cells) were chose to transplant under the armpit of approximately 18-20 g female BALB/c mice for 21 days. For imaging of tumor, the mice were treated with **NLAP** (50 μL, 100 μM, 0-30 min) by spraying way in the presence of bestatin (0 and 200 μM, 1 h) for *in vivo* imaging. The *in vivo* (living mice) imaging was carried out using the FUSION FX imaging system (Vilber Lourmat). Excitation wavelength was 640 nm. The emission wavelength was at 695 nm.

7. Supplementary Figures

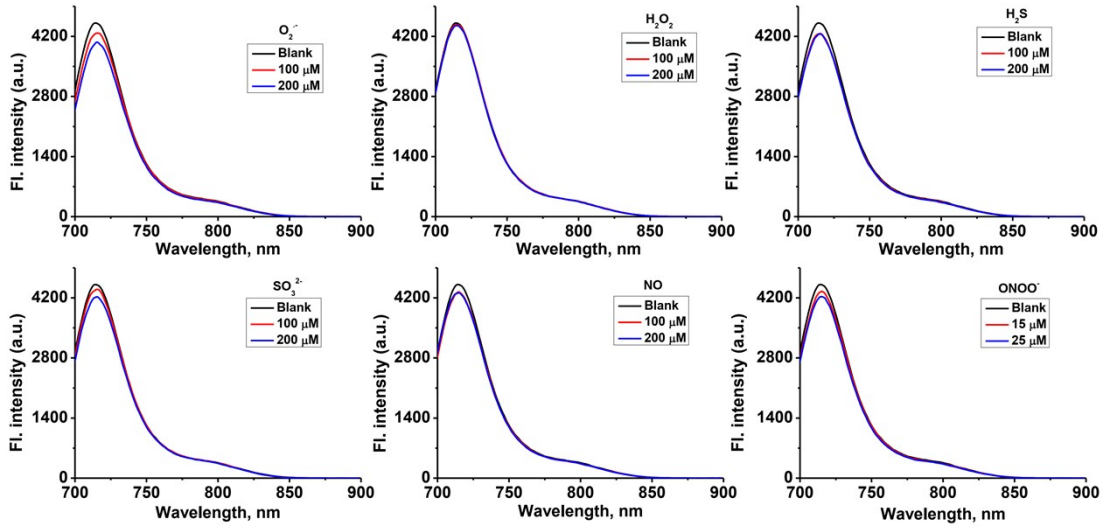

Fig. S1 Fluorescence spectra of compound **NOH** (5 μM) treated with various agents in pH 7.4 PBS/DMSO (v/v, 9/1) buffer solution. $O_2^{\bullet-}$, H_2O_2, H_2S, SO_3^{2-}, NO: 0-200 μM; $ONOO^-$: 0-25 μM.

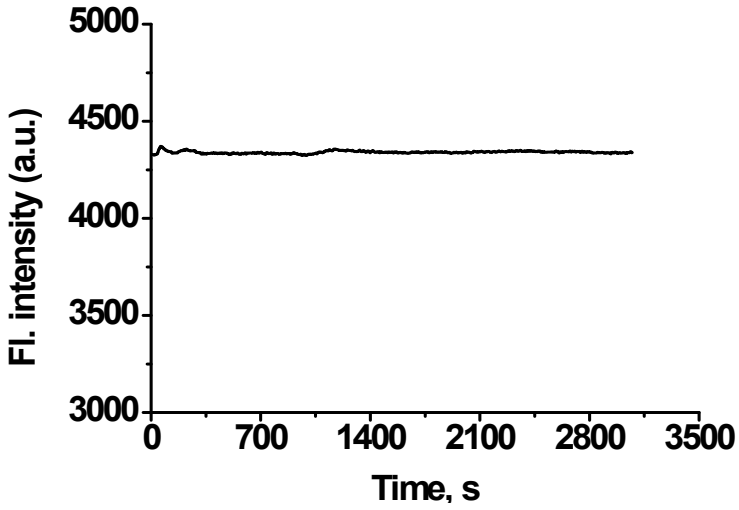

Fig. S2 Fluorescence intensity of **NOH** (5 μM) with the different time (0-50 min) in pH 7.4 PBS/DMSO (v/v, 9/1) buffer solution. λ_{ex} = 680 nm.

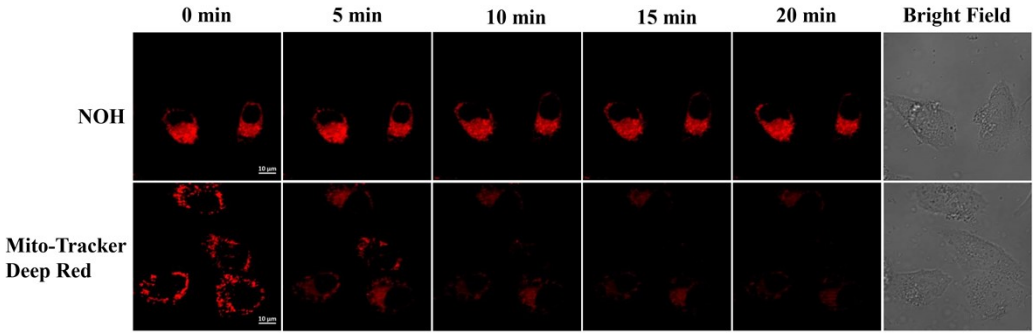

Fig. S3 Confocal fluorescence images of living HepG2 cells cultured with **NOH** and Mito-Tracker Deep Red with continuous irradiation using confocal microscope with the same parameters. Scale bar = 10 μm. λ_{ex} = 633 nm, λ_{em} = 658-747 nm.

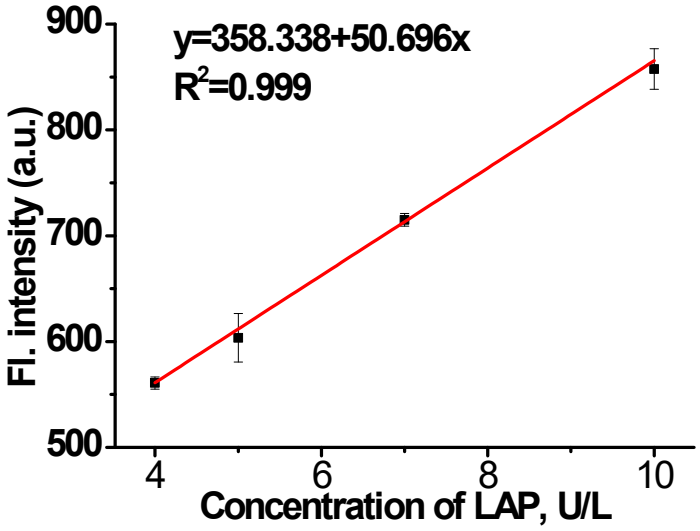

Fig. S4 A linear relationship exists between the intensity of fluorescence and the concentration of LAP.

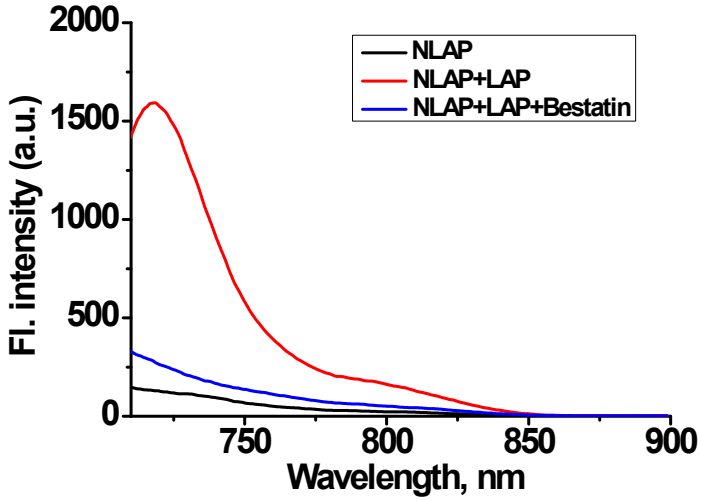

Fig. S5 Effect of bestatin on LAP activity. Black trace: **NLAP** only (5 μM); red trace: **NLAP** (5 μM) + LAP (50 U/L); blue trace: **NLAP** (5 μM) + LAP (50 U/L) + bestatin (100 μM). λ_{ex} = 680 nm.

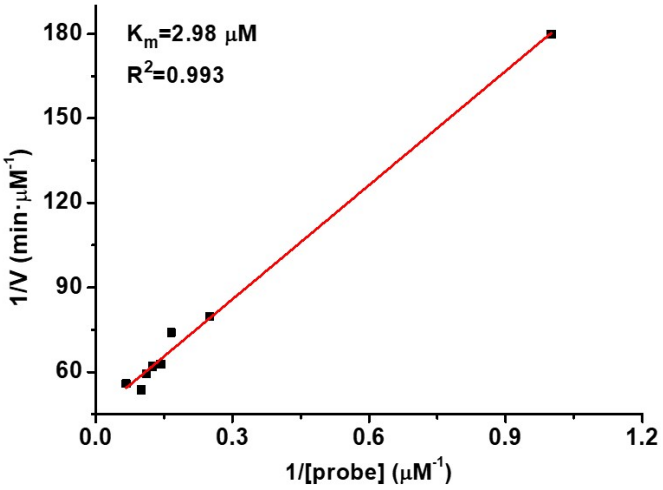

Fig. S6 Lineweaver-Burk plot for the enzyme-catalyzed reaction. The Michaelis-Menten equation was described as: $V = V_{max}$ [probe]/(K_m+[probe]), where V is the initial reaction rate, [probe] is the probe concentration (substrate), and the K_m is the Michaelis constant. Conditions: 80 U/L LAP, 1-15 μM of **NLAP**. The measurements were performed at 37 °C with 680 nm excitation wavelength.

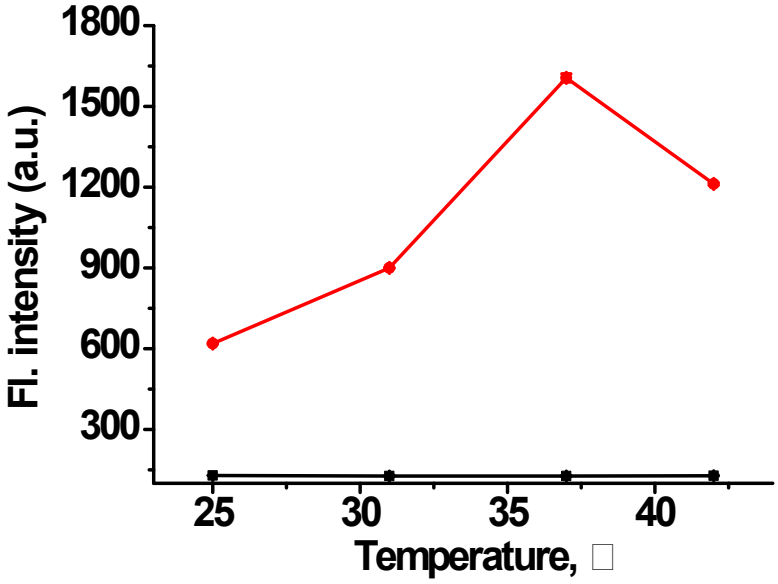

Fig. S7 The effect of temperature on the catalytic activity of LAP. Black trace: only **NLAP** (5 μM); Red trace: **NLAP** (5 μM) + LAP (50 U/L). $\lambda_{ex}/\lambda_{em}$ = 680/717 nm.

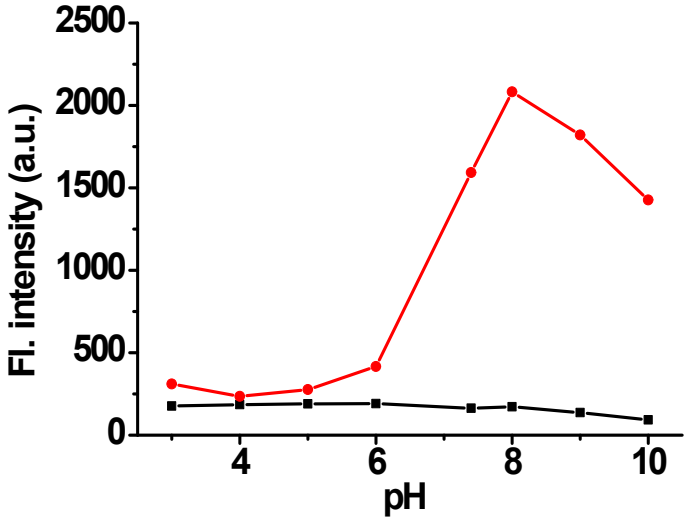

Fig. S8 Effects of pH on the fluorescence of probe **NLAP** (5 μM) reacting with LAP (50 U/L). $\lambda_{ex}/\lambda_{em}$ = 680/717 nm.

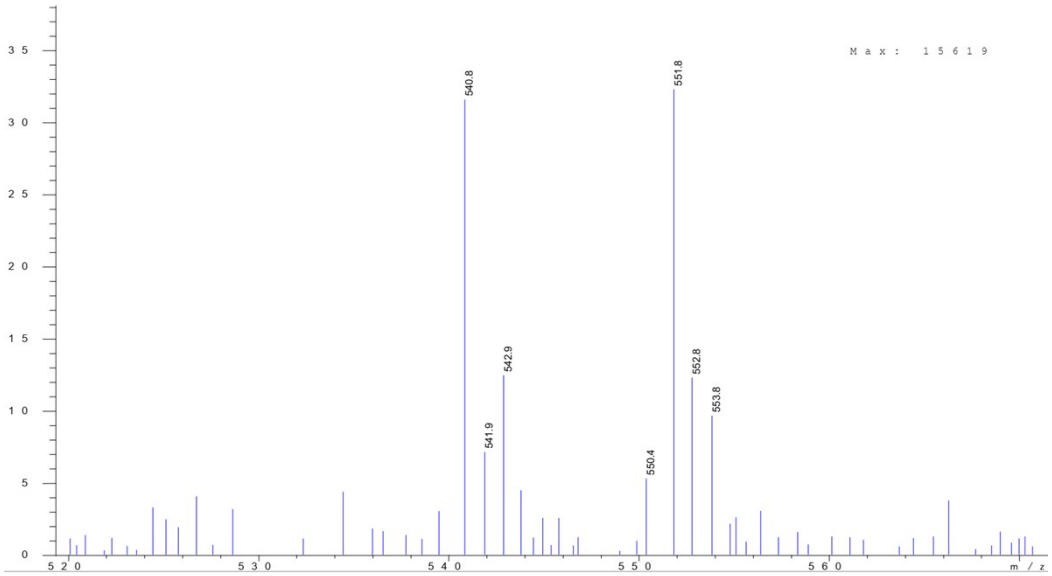

Fig. S9 The ESI mass spectrum of compound **NLAP** in the presence of LAP.

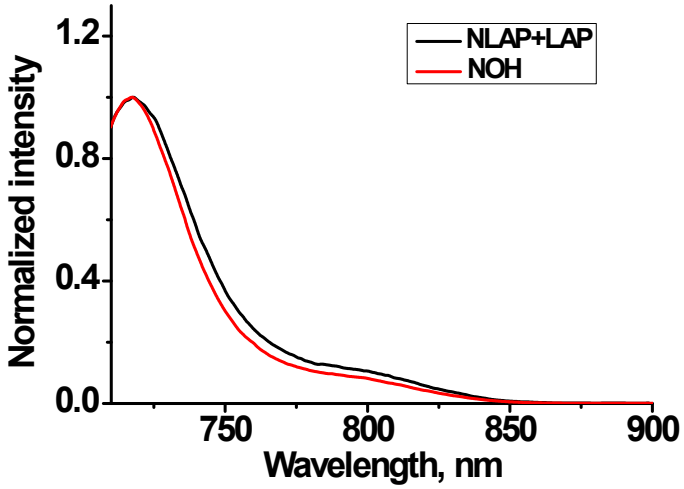

Fig. S10 Normalized absorption of **NLAP** (5 μM) in the presence of LAP (50 U/L) and **NOH** (5 μM).

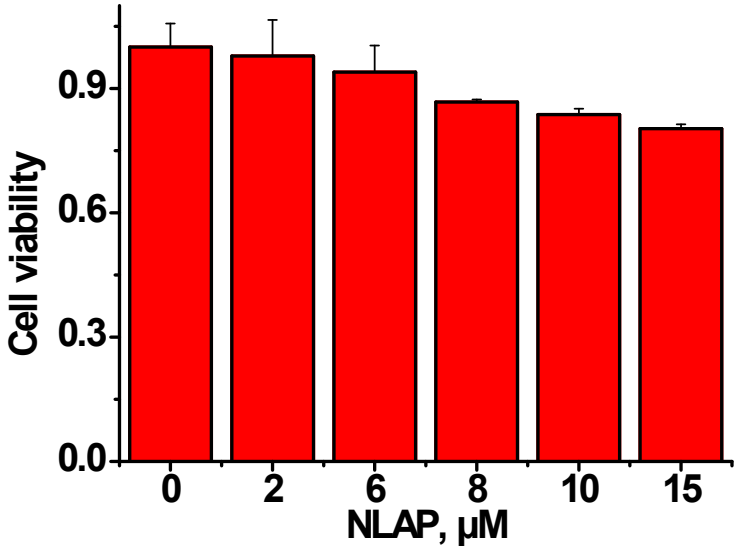

Fig. S11 Cytotoxicity of **NLAP** for HepG2 cells. Cells were incubated with the probe at corresponding concentrations for 24 h. Cell viability was measured by MTT assay and the results are reported as percentage relative to untreated cells (mean ± SD).

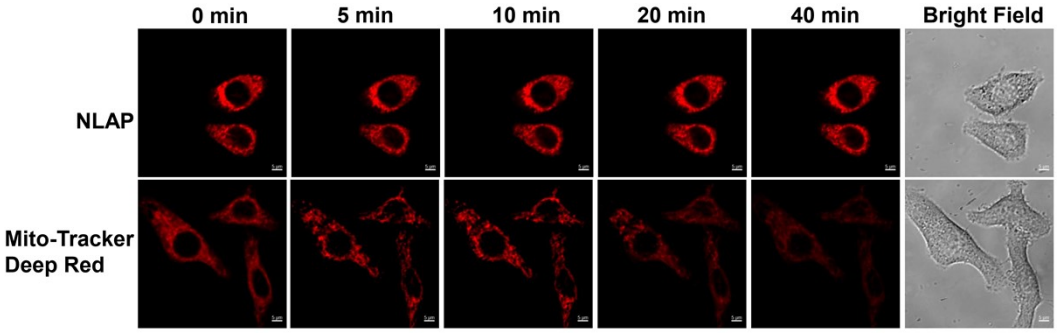

Fig. S12 Confocal fluorescence images of living HepG2 cells cultured with **NLAP** and Mito-Tracker Deep Red with continuous irradiation using confocal microscope with the same parameters. Scale bar = 5 μm. λ_{ex} = 633 nm, λ_{em} = 658-747 nm.

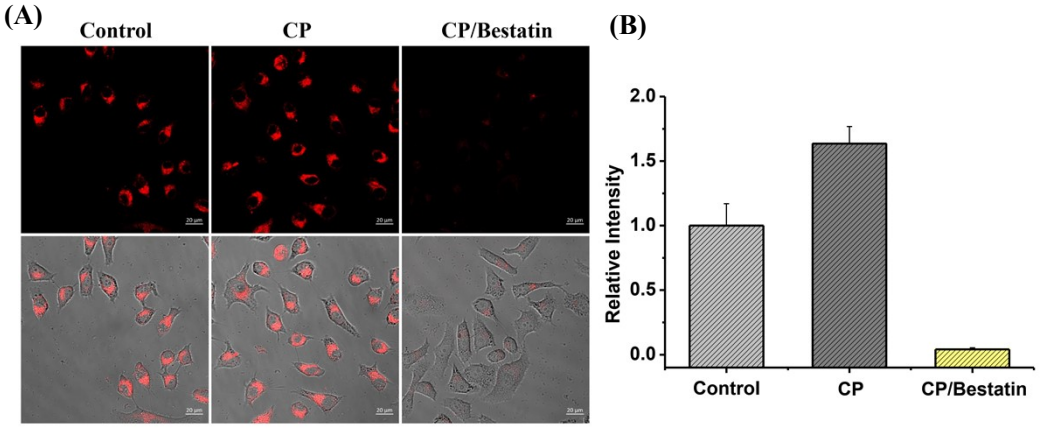

Fig. S13 (A) Fluorescent imaging of LAP through different drugs treatment. Cells were treated with CP (0 and 2 mg/L, 12 h) in the presence of bestatin (0 and 200 μM, 1 h), then incubated by **NLAP** (5 μM) for 30 min. (B) The relative fluorescence intensity in A. λ_{ex} = 633 nm, λ_{em} = 658-747 nm. Scale bar = 20 μm.

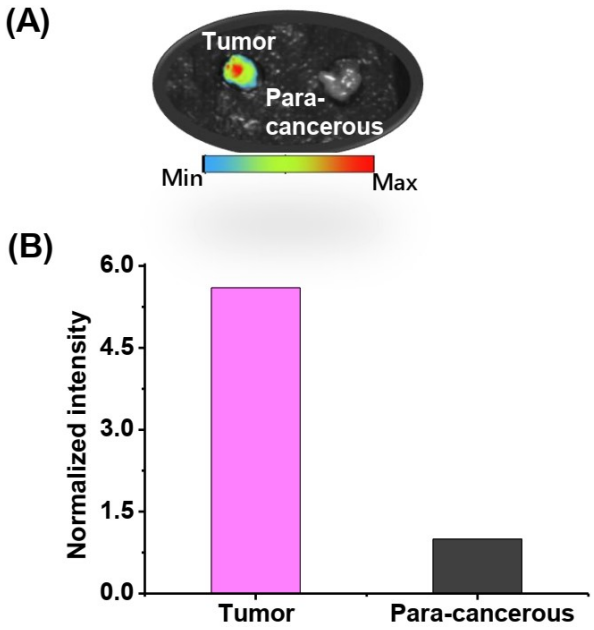

Fig. S14 (A) Fluorescence imaging of the tumor tissue and para-carcinoma tissue *in vitro* by spraying **NLAP** (50 μL, 100 μM). λ_{ex} = 640 nm, λ_{em} = 695 nm. (B) Normalized fluorescence intensities in A.

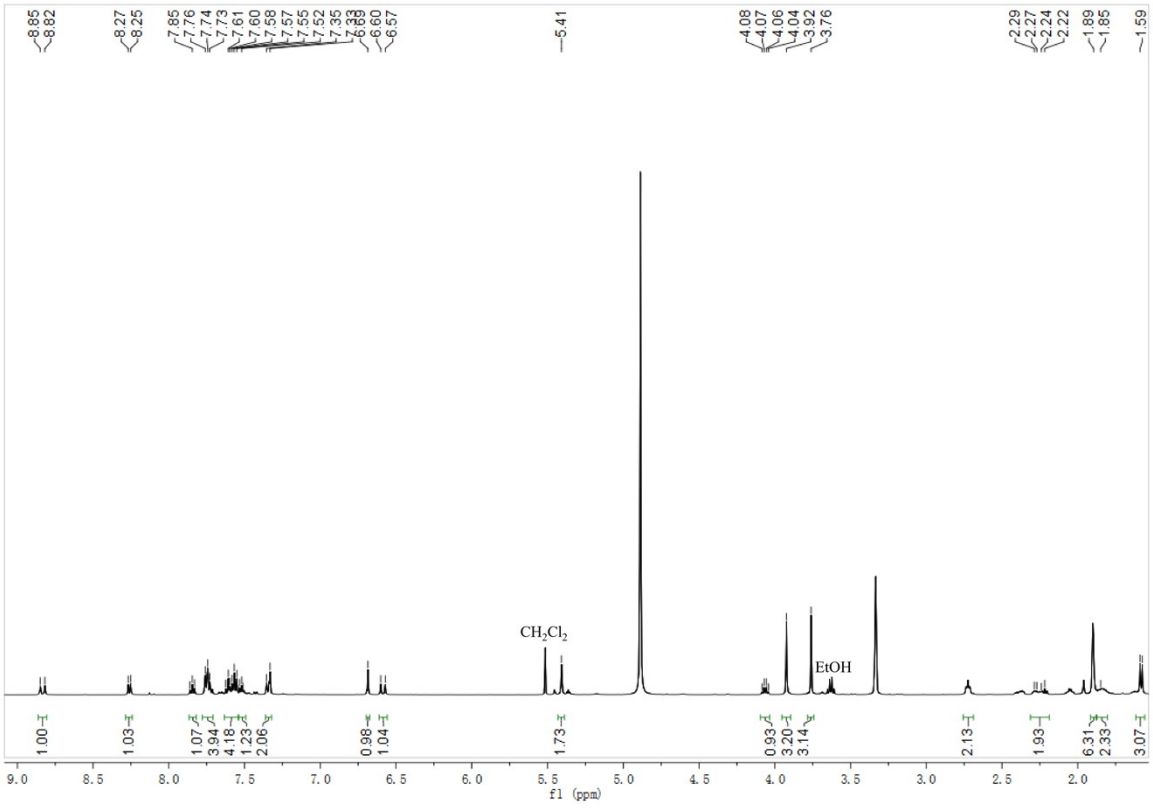

Fig. S15 ^{1}HNMR spectra of **NLAP** in CD_3OD.

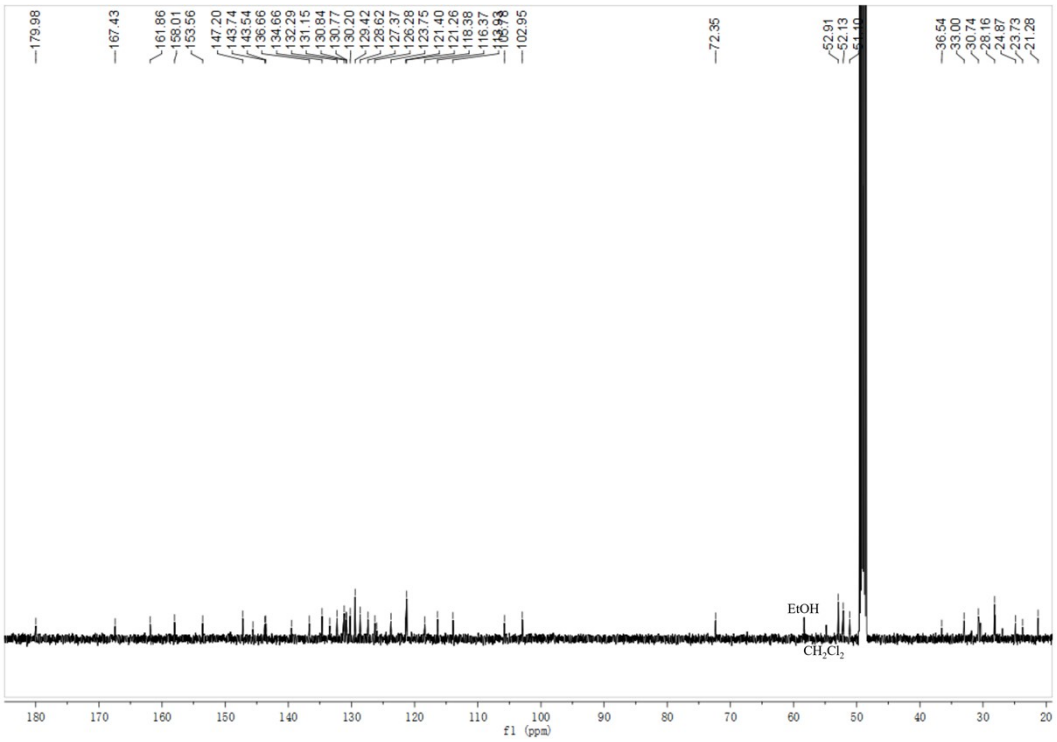

Fig. S16 ^{13}CNMR spectra of **NLAP** in CD_3OD.

8. References

1 R. Zhong, R. Jiang, J. Zeng, X. Gong, X. Yang, L. He, L. Yuan and D. Cheng, *Anal. Chem.,* **2023**, *95*, 2428–2435.