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Supporting Information

Selectively Light-Up Hydrogen Peroxide in Hypoxic Cancer Cells with a Novel Fluorescent Probe

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Experimental Procedures

Materials and instrumentation.

Chemicals were purchased from Energy-Chemical, Sigma-Aldrich, J&K and used without further purification. Solvents and other common reagents were obtained from Sigma-Aldrich. ^{1}H NMR and ^{13}C NMR spectra were measured on a Bruker ARX 400 MHz spectrometer. High-resolution mass spectra (HRMS) were recorded on a GCT Premier CAB 048 mass spectrometer operating in MALDI-TOF mode. UV-vis absorption spectra were recorded on a Rarian 50 Conc UV-Visible spectrophotometer. Fluorescence emission spectra were recorded on a Hitachi F-4500 fluorescence spectrophotometer. Confocal fluorescence imaging experiments were performed with an Zeiss LSM 800 laser scanning microscopy system.

General procedures for the detection of H_2O_2

Unless otherwise noted, all the spectral measurements were performed in 5 mM phosphate buffer (pH 7.4) according to the following procedure. The stock solution (1.0 mM) of probe HCyHP was first prepared in DMSO. 20 μL of HCyHP stock solution was added to 2 mL PBS followed by addition of different volume of H_2O_2 solution. The mixture was incubated for certain times at 37 ℃ and then, the reaction solution was transferred to a quartz cell with 1-cm optical length for measurements. In the meantime, the blank solution without H_2O_2 was also prepared and measured under the same conditions for comparison.

Hydrogen peroxide (H_2O_2), *tert*-butyl hydroperoxide (TBHP), and hypochlorite (ClO^-) were delivered from 30%, 70%, and 5% aqueous solutions, respectively. Hydroxyl radical (•OH) was generated by reaction of 100 μM Fe^{2+} with 100 μM H_2O_2. ROO• was generated from 2,2'-azobis(2-amidinopropane)dihydrochloride (AAPH), which was dissolved in deionizer water and then stirred at 25 ℃ for 30 min. Theoretically, 1 equiv of AAPH will generate 2 equiv of ROO•. R stands for 2-amidinoisopropyl. $O_2^{\bullet-}$ was sourced from KO_2. The powder of KO_2 was directly added into the probe solution. C[KO_2] = 2 mM. Nitric oxide was used as DEA NONOate which was source of NO. In titration experiments, each time a 2 ml solution of ligand was filled in a quartz cuvette (path length, 1 cm) and spectra were recorded after the addition of appropriate analyte.

Determination of the detection limit of HCyHP toward addition of H_2O_2

Based on the linear fitting in Figure 1B and Figure 2B, the detection limit (*C*) is estimated as follows:

$$C = 3\sigma/B$$

Where σ is the standard deviation obtained from three individual absorption ratio (A_{580}/A_{476}) or fluorescence measurements (I_{599}) of HCyHP (10 μM) without any H_2O_2 and *B* is the slope obtained after linear fitting the titration curves within certain ranges.

Cell cultures

The HeLa cells were cultured in DMEM (containing 10% heat-inactivated FBS, 100 mg·mL^{-1} penicillin and 100 mg·mL^{-1} streptomycin) at 37 ℃ in a humidified incubator with 5% CO_2. Before the experiments, the cells were pre-cultured until confluence was reached.

Cell viability

Cell viability was determined by using MTT assay which is based on the reduction of 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, yellow in color) into formazan (blue color) by mitochondrial succinate dehydrogenase. Dispense 100 μL of cell suspension (5000 cells/well) in a 96-well plate. Pre-incubate the plate for 24 h at 37 ℃ in a humidified incubator with 5% CO_2. Add 10 μL of various concentrations of HCyHP into the culture media in the plate. Incubate the plate for 24 h in the incubator. 100 μL of freshly prepared MTT solution was added in each well. Medium was removed after the incubation period of 2-4 hours followed by the addition of 200 μL of DMSO to dissolve the formazan crystals. Absorbance was taken at 595 nm by an ELISA Plate Reader (Biotek Synergy HT). Untreated cells were taken as control. All the experiments were performed in triplicate. of compounds was determined by using given formula:

$$\%\ \text{Cell viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of untreated cells}} \times 100$$

Cell treatment and cell imaging

For the purpose of this study, the following groups of HeLa cells were chosen for cell imaging:

1) For the detection of exogenous H_2O_2, the cells in control group were exposed to probe HCyHP (5 μM) for 15 min. The cells in experimental group were pre-stained with HCyHP (5 μM) for 15 min and further treated with H_2O_2 (200 μM) for another 30 min.
2) For the detection of endogenous H_2O_2, the cells in control group were exposed to probe HCyHP (5 μM) for 60 min. The cells in experimental group were stimulated with LPS (3.0 μg/mL for

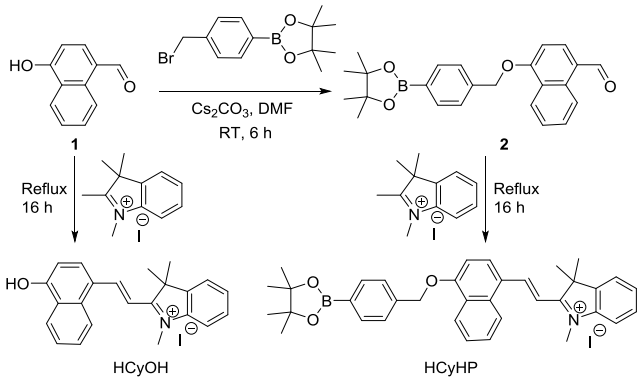
24 h) and then exposed to probe HCyHP (5 μM) for 60 min.

3) For the detection of hypoxia, HeLa cells in both control group and experimental group were stained with HCyHP (5 μM) for 15 min. Then the cells in control group were incubated under normoxic conditions for 10 h. The cells in the other group were incubated under hypoxic conditions (< 1% O_2) for 10 h.

For cell imaging, the cells were washed with PBS three times and were imaged with a Zeiss LSM 800 laser scanning microscopy system. A 561 nm laser was used as the light source and emission was collected from 580 to 700 nm.

***In vivo* animal imaging**

Female BALB/c nude mice (4–6 weeks old and weighted 15–20 g) were purchased from Vital River Laboratory Animal Technology Co. Ltd (Beijing, China) and all animals received care incompliance with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals. The procedures were approved by Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences Animal Care and Use Committee. To set up the tumor model, 4T1 cells (1×10^6) were administered by subcutaneous injection into the right flanks of the mice. Tumor-bearing mice were used 2 weeks post-tumor inoculation. When the tumor volumes reached about 50 mm^3, the probe solution (1 mM in DMSO, 10 μL) was injected to nude mice via intratumoral injection. Control group was injected with 10 μL of DMSO. Images were taken 20 min after injection using the Maestro *in vivo* imaging system.



Scheme S1. Synthetic routes of HCyOH and HCyHP.

Synthesis of compound 2

To a solution of 4-hydroxy-1-naphthaldehyde (compound **1**, 172.2 mg, 1.0 mmol) and 2-[4-(bromomethyl)phenyl]-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (297.0 mg, 1.0 mmol) in 30 mL DMF, was added caesium carbonate (488.7 mg, 1.5 mmol). The mixture was stirred at room

temperature for 6 h under the nitrogen atmosphere. After concentrated, the residue was purified by silica gel chromatography with petroleum ether/ethyl acetate (15:1 v/v) to give compound **2** as a white solid (232.9 mg, 60%). ^{1}H NMR (400 MHz, DMSO-d_6), δ 10.19 (s, 1H), 9.23 (d, J = 8.5 Hz, 1H), 8.34 (d, J = 8.4 Hz, 1H), 8.15 (d, J = 8.0 Hz, 1H), 7.75 (d, J = 7.7 Hz, 3H), 7.66 (t, J = 7.7 Hz, 1H), 7.59 (d, J = 7.5 Hz, 2H), 7.30 (d, J = 8.1 Hz, 1H), 1.30 (s, 12H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 193.20, 159.41, 140.47, 140.04, 135.17, 131.66, 129.99, 127.37, 127.07, 125.36, 124.92, 124.76, 122.64, 105.86, 84.18, 70.46, 25.14. mp 163 ^{o}C – 165 ^{o}C.

Synthesis of HCyHP

A mixture of compound **2** (388.2 mg, 1.0 mmol), 1,2,3,3-tetramethyl-3H-indol-1-ium iodide (301.2 mg, 1.0 mmol) in ethanol was refluxed under nitrogen atmosphere for 16 h. After cooling to room temperature, ethyl ether was added and HCyHP was precipitated and collected as a red solid (604.3 mg, 90%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.96 (d, J = 16.0 Hz, 1H), 8.62 (d, J = 8.5 Hz, 1H), 8.45 (d, J = 8.6 Hz, 1H), 8.39 (d, J = 8.5 Hz, 1H), 7.94 – 7.88 (m, 2H), 7.86 – 7.81 (m, 1H), 7.80 – 7.67 (m, 4H), 7.67 – 7.54 (m, 4H), 7.36 (d, J = 8.5 Hz, 1H), 4.18 (s, 3H), 1.87 (s, 6H), 1.31 (s, 12H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 181.84, 159.21, 148.70, 143.71, 142.41, 140.14, 135.20, 134.84, 132.98, 130.65, 129.59, 129.45, 127.39, 127.05, 125.44, 124.02, 123.60, 123.35, 123.09, 115.45, 112.91, 107.27, 84.20, 70.55, 52.48, 34.89, 26.40, 25.42, 25.15. HRMS (MALDI-TOF): m/z: $[M\text{-}I]^+$ calcd for $C_{36}H_{39}BNO_3^+$: 544.3018; found: 544.3018. mp 142 ^{o}C – 144 ^{o}C.

Synthesis of HCyOH

A mixture of compound **1** (172.2 mg, 1.0 mmol), 1,2,3,3-tetramethyl-3H-indol-1-ium iodide (301.2 mg, 1.0 mmol) in ethanol was refluxed under nitrogen atmosphere for 16 h. After cooling to room temperature, ethyl ether was added and compound HCyOH was precipitated and collected as a purple solid (432.3 mg, 95%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.94 (d, J = 15.8 Hz, 1H), 8.62 (d, J = 8.4 Hz, 1H), 8.43 (d, J = 8.6 Hz, 1H), 8.31 (d, J = 8.3 Hz, 1H), 7.88 (t, J = 7.2 Hz, 2H), 7.80 (t, J = 7.7 Hz, 1H), 7.72 – 7.53 (m, 4H), 7.13 (d, J = 8.4 Hz, 1H), 4.14 (s, 3H), 1.86 (s, 6H). ^{13}C NMR (100 MHz, DMSO-d_6), δ 181.35, 161.03, 148.72, 143.47, 142.43, 133.83, 131.71, 129.66, 129.41, 129.18, 126.33, 124.88, 123.69, 123.28, 123.19, 122.00, 115.11, 110.90, 109.92, 52.16, 34.69, 26.74. HRMS (MALDI-TOF): m/z: $[M\text{-}I]^+$ calcd for $C_{23}H_{22}NO^+$: 328.1696; found: 328.1666. mp 255 ^{o}C – 257 ^{o}C.

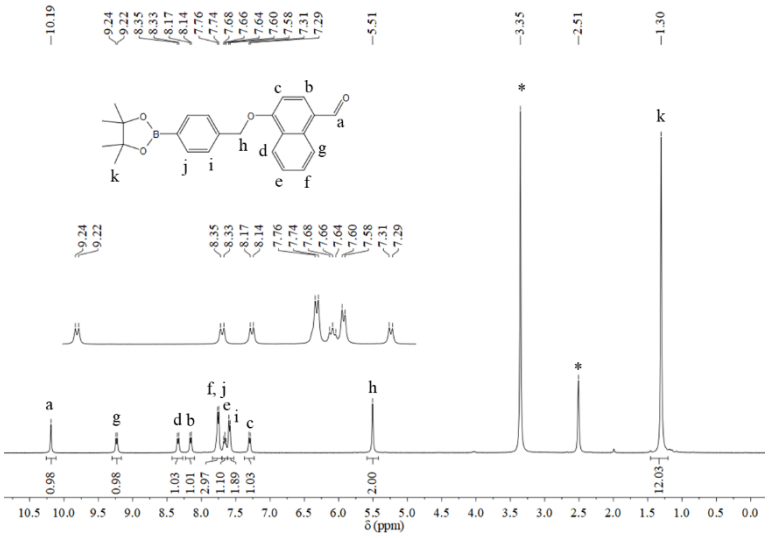


Fig. S1. ^{1}H NMR spectrum of compound **2** in DMSO-d_6.

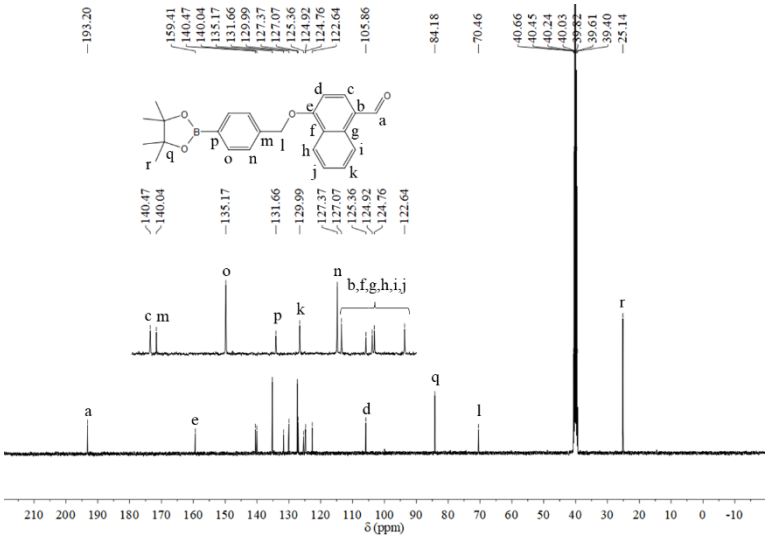


Fig. S2. ^{13}C NMR spectrum of compound **2** in DMSO-d_6.

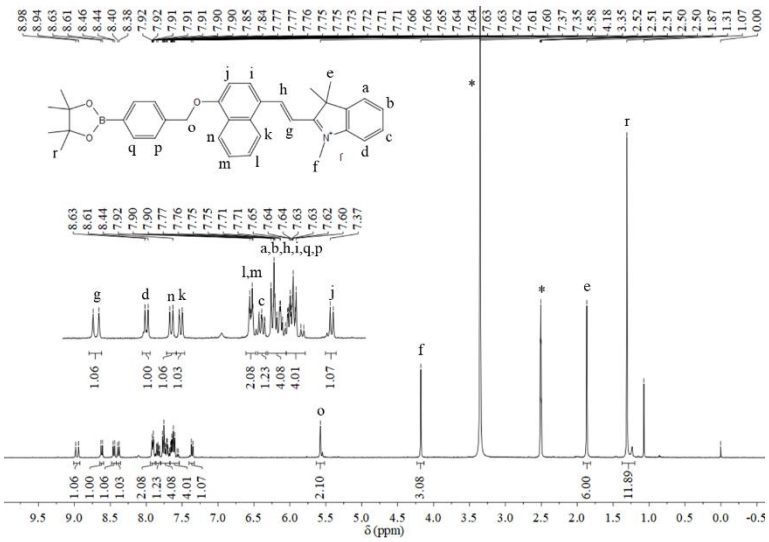


Fig. S3. ^{1}H NMR spectrum of HCyHP in DMSO-d_6.

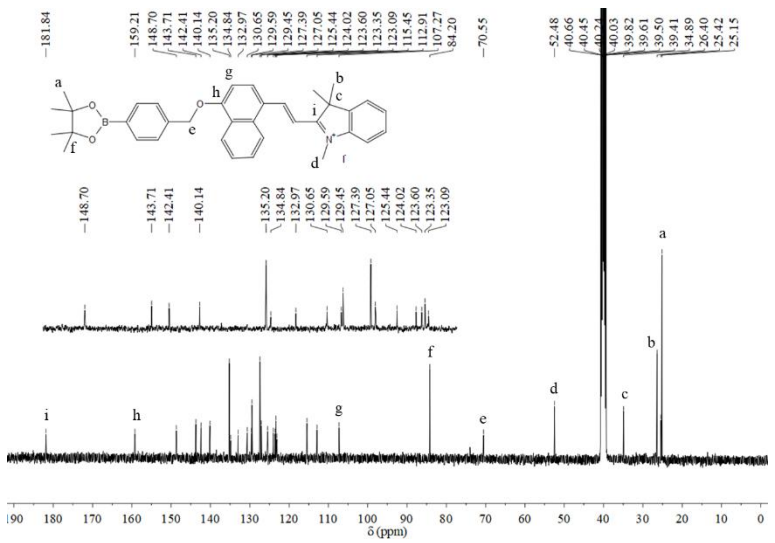


Fig. S4. ^{13}C NMR spectrum of HCyHP in DMSO-d_6.

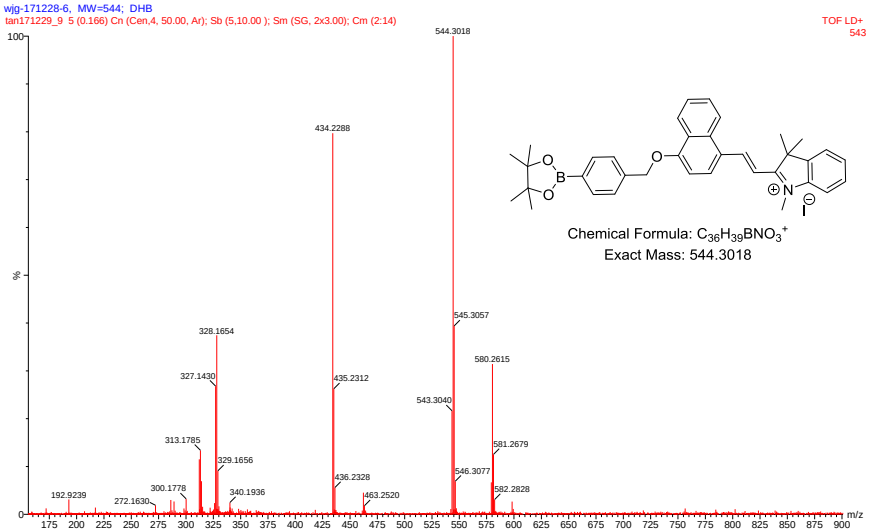


Fig. S5. HR-MS spectrum of HCyHP.

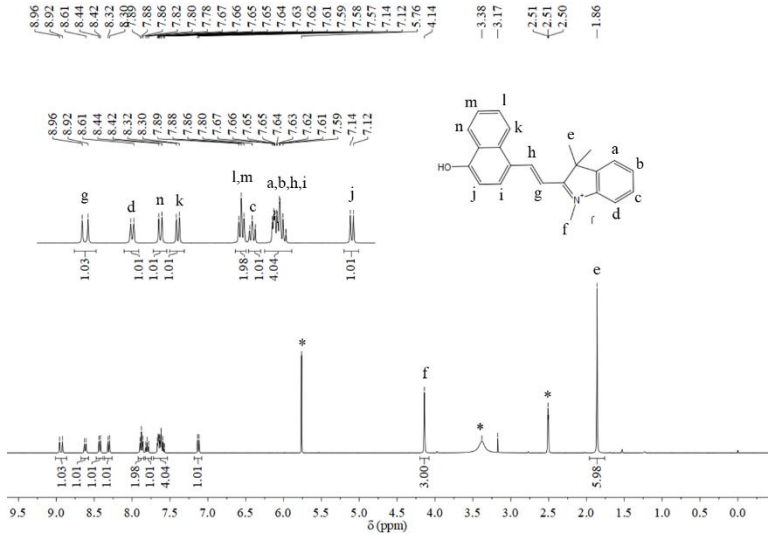


Fig. S6. ^{1}H NMR spectrum of HCyOH in DMSO-d_6.

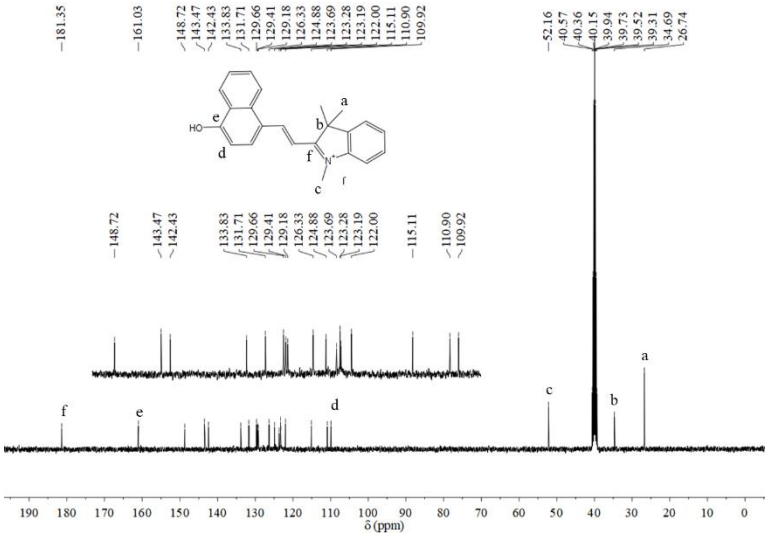


Fig. S7. ^{13}C NMR spectrum of HCyOH in DMSO-d_6.

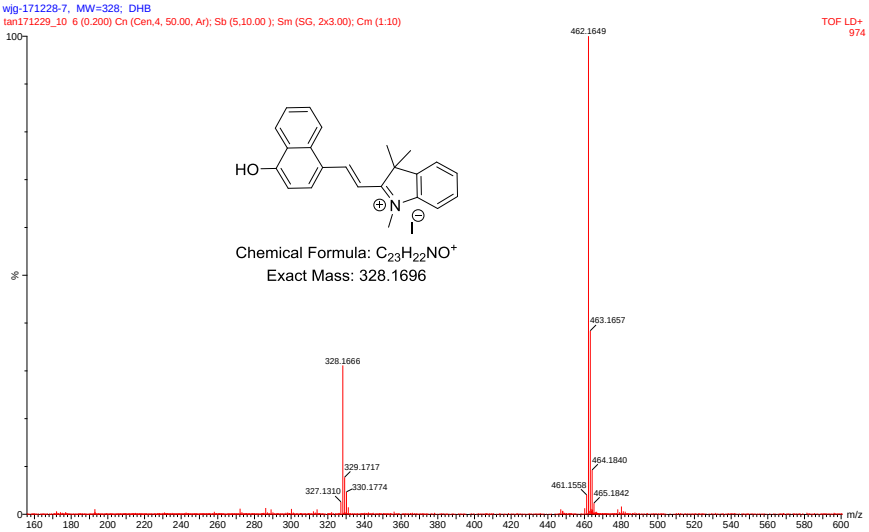


Fig. S8. HR-MS spectrum of HCyOH.

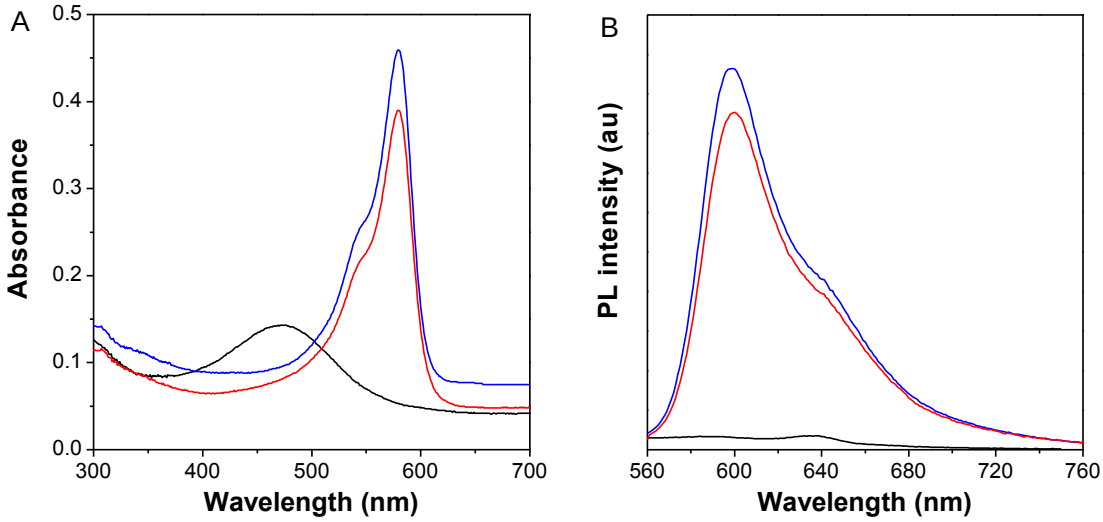


Fig. S9. (A) UV/Vis spectra and (B) fluorescence spectra of HCyHP (5 μM, black), HCyOH (5 μM, blue) and HCyHP (5 μM, red) after incubation with H_2O_2 (125 μM) at 37 ℃ for 15 min in PBS solution.

Fig. S10. HRMS spectrum of HCyHP after incubation with H_2O_2 (125 μM) at 37 ℃ for 15 min.

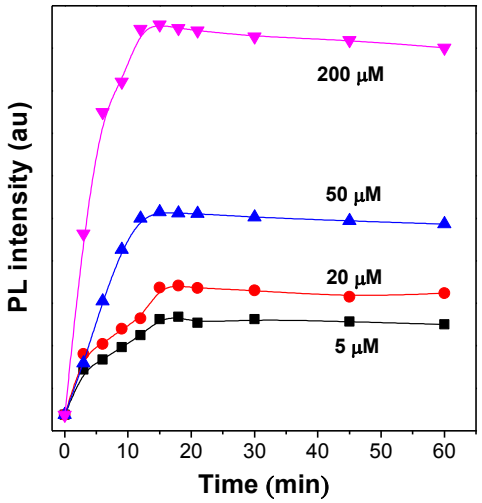


Fig. S11. The fluorescence intensity of HCyHP at 599 nm in the presence of different concentrations of H_2O_2 as a function of incubation time.

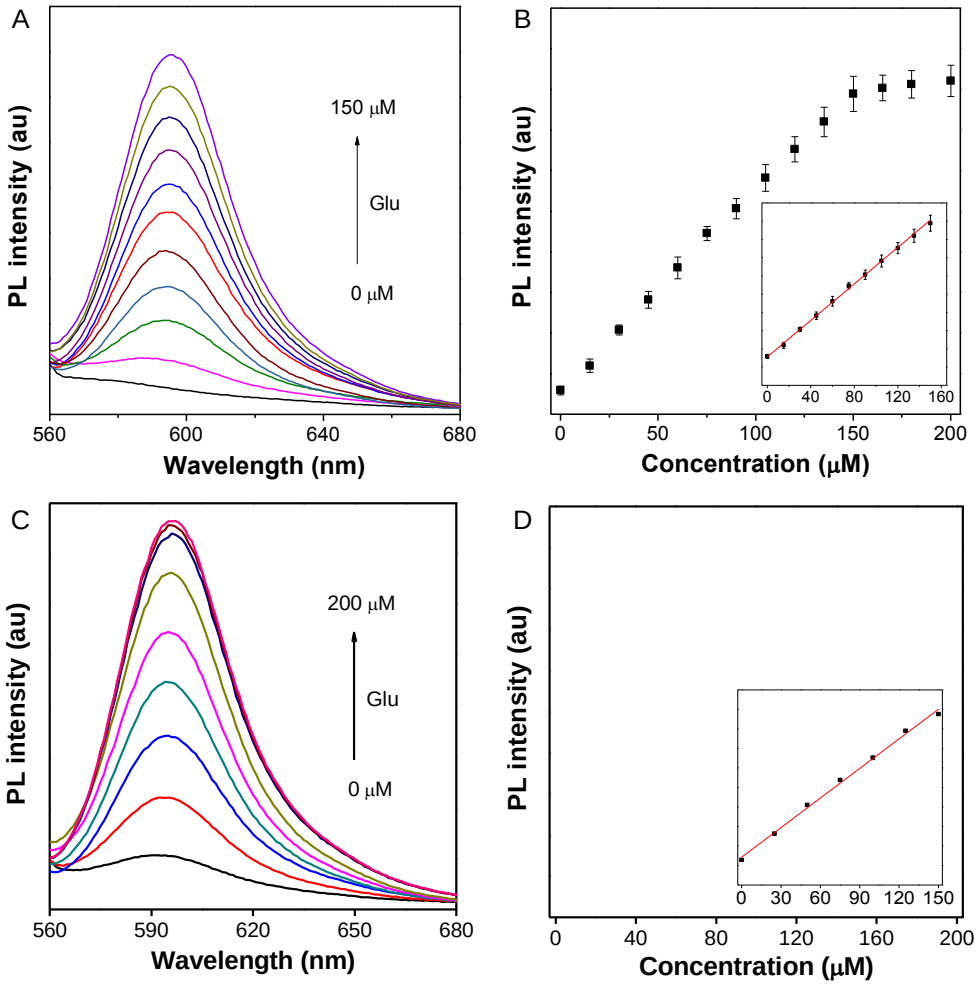


Fig. S12. (A) Fluorescence spectra of HCyHP (5 μM) in PBS after incubation with GOx (1.5 U/mL) and different concentrations of D-glucose at 37 ^{0}C for 50 min. (B) The fluorescence intensity of HCyHP at 599 nm as a function of the concentration of D-glucose (0-200 μM). Inset showed the plot and linear fitting of fluorescence intensity at 599 nm *vs*. the concentration of D-glucose (0-150 μM). Y=1.233X+14.959, R^2=0.998. λ_{ex} = 524 nm. (C) Fluorescence spectra of HCyHP (5 μM) in PBS containing 0.5% FBS after incubation with GOx (1.5 U/mL) and different concentrations of D-glucose at 37 ^{0}C for 50 min. (D) The fluorescence intensity of HCyHP at 599 nm as a function of the concentration of D-glucose (0-200 μM). Inset showed the plot and linear fitting of fluorescence intensity at 599 nm *vs*. the concentration of D-glucose (0-150 μM). Y=1.263X+35.951, R^2=0.996. λ_{ex} = 524 nm.

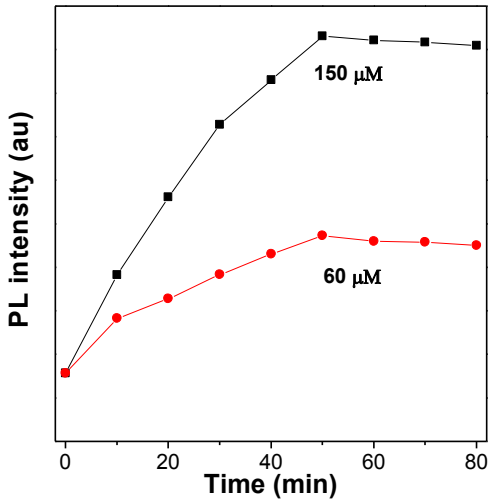


Fig. S13.The fluorescence intensity of HCyHP (5 μM) at 599 nm incubated with GOx (1.5 U/mL) and different concentrations of D-glucose as a function of time.

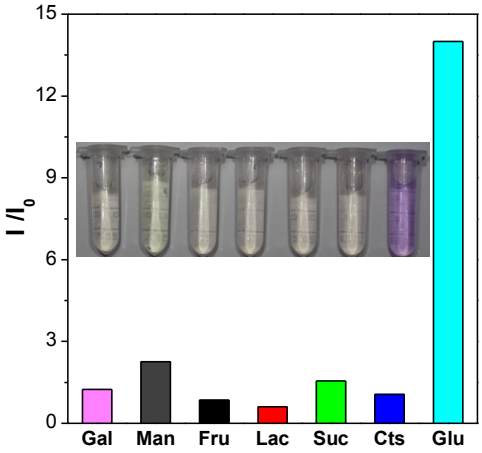


Fig. S14. Variation of the relative fluorescence intensity (I/I_0) at 599 nm of HCyHP (5 μM) after incubation with GOx (1.5 U/mL) and 150 μM of glucose (Glu) or excess galactose (Gal, 1 mM), mannose (Man, 1 mM), fructose (Fru, 1 mM), lactose (Lac, 0.5 mM), sucrose (Suc, 0.5 mM) and chitosan (Cts, 1 mM). The reaction was performed at 37 ℃ for 50 min in 5 mM PBS at pH 7.4. λ_{ex} = 524 nm. Inset: photographs of solutions incubated with different saccharides under ambient light.

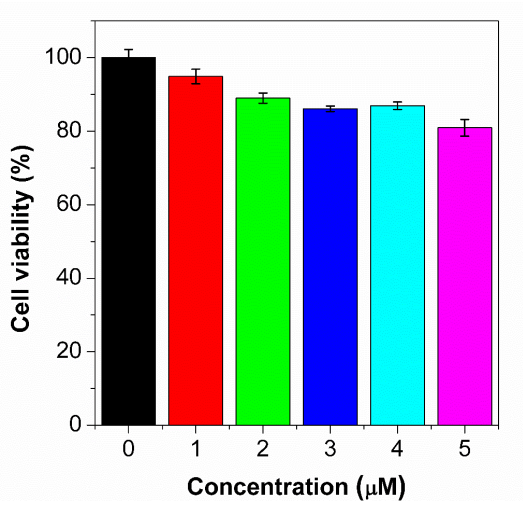


Fig. S15. Cell viability of HeLa cells at varied concentrations of HCyHP using MTT method.

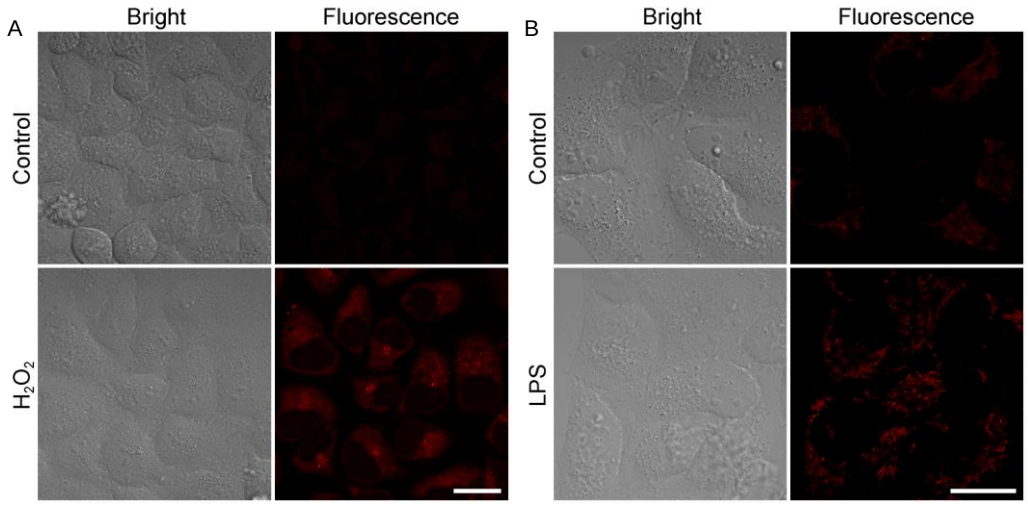


Fig. 16. (A) HeLa cell imaging of HCyHP (5 μM for 15 min at 37 ℃) in the absence or presence of H_2O_2 (200 μM for 30 min). (B) HeLa cell imaging of HCyHP (5 μM for 60 min at 37 ℃) with or without treatment of LPS (3.0 μg/mL for 24 h). Images were acquired using 561 nm laser excitation and fluorescent emissions were collected from 580 to 700 nm. Scale bar: 20 μm.

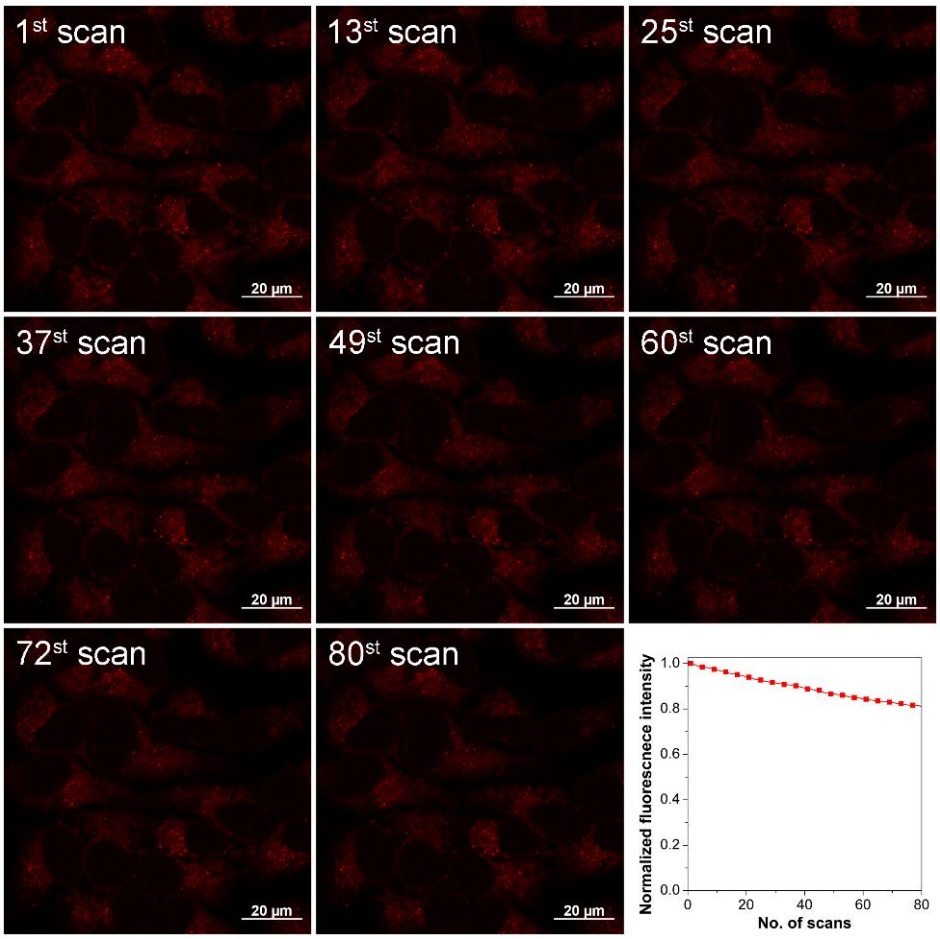


Fig. S17. Loss in fluorescence of HeLa cells stained with HCyHP continuously irradiated with 561 nm laser at different scans. The image was scaned very 3.58 s.

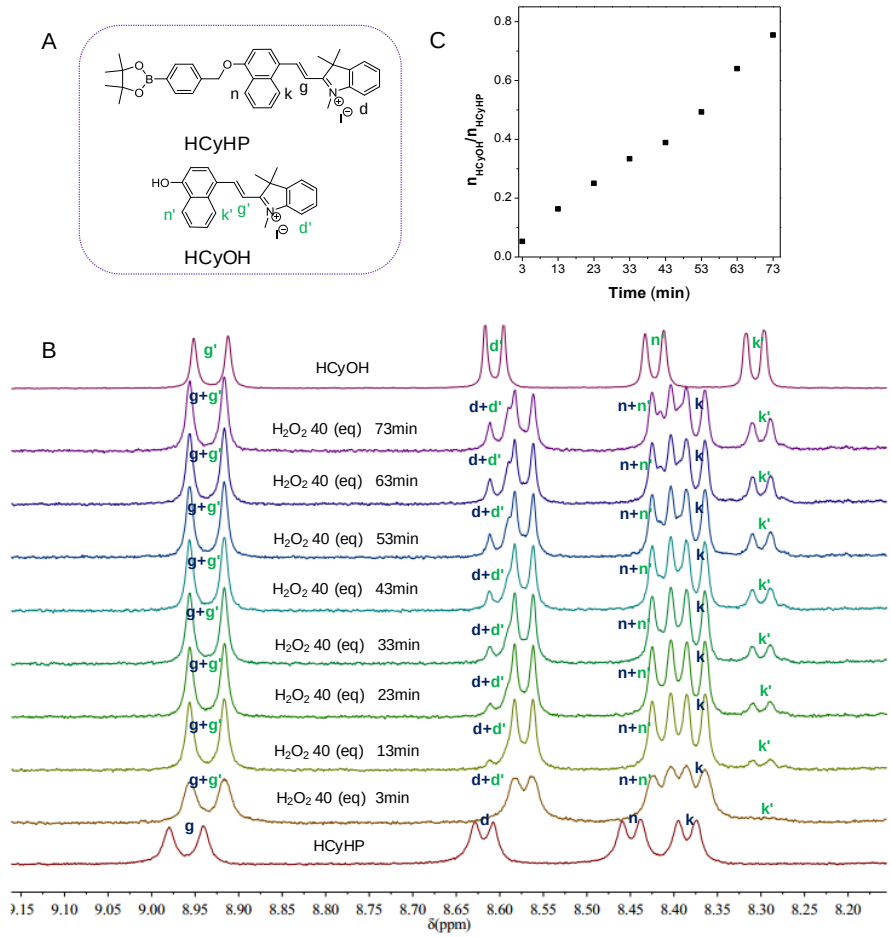


Fig. S18. (A) The chemical structure of HCyHP and HCyOH. (B) ^{1}H NMR spectra of HCyHP in the presence of 40.0 equivalents H_2O_2 incubated at room temperature for different time in DMSO-d_6 (The concentration of HCyHP is 14.9 mmol/L). (C) The molar ratios of HCyOH and HCyHP in the presence of 40.0 equivalents of H_2O_2 as a function of the incubation time according to the NMR data in (B).

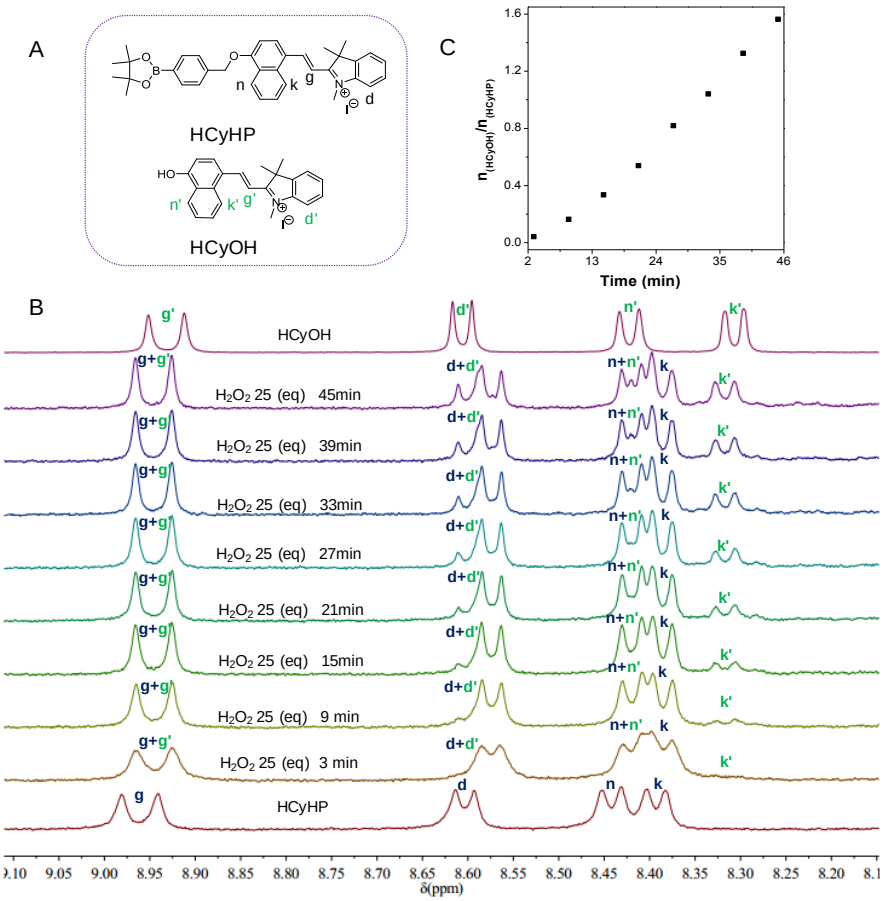


Fig. S19. (A) The chemical structure of HCyHP and HCyOH. (B) ^{1}H NMR spectra of HCyHP in the presence of 25.0 equivalents H_2O_2 incubated at 37 ^{o}C for different time in DMSO-d_6 (The concentration of HCyHP is 14.9 mmol/L). (C) The molar ratios of HCyOH and HCyHP in the presence of 25.0 equivalents of H_2O_2 as a function of the incubation time according to the NMR data in (B).

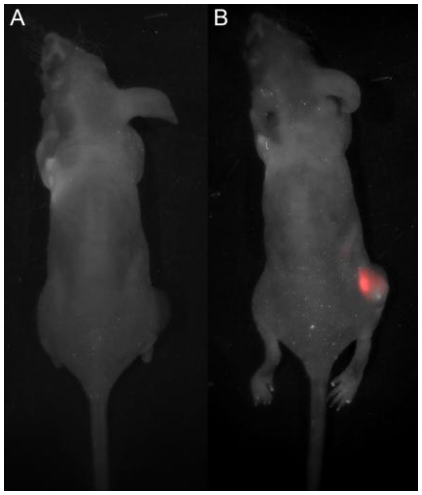


Fig. S20. *In vivo* fluorescence imaging of H_2O_2 in the tumor-bearing live mice using HCyHP 20 min after injection. (A) Control group: only 10 μL DMSO was injected. (B) Experimental group: 10 μL probe HCyHP (1 mM) in DMSO solution was injected.