Supporting Information

Revealing the signaling regulation of hydrogen peroxide to cell pyroptosis by a ratiometric fluorescent probe in living cells

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Experimental Procedures

1. Materials and apparatus

4-(bromomethyl)-benzeneboronic acid pinacol ester, terephthalaldehyde, palmitic acid (PA), piperidine and 4-methylquinoline were purchased from Aladdin. 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), phorbol 12-myristate 13-acetate (PMA), *N*-acetyl-cysteine (NAC), PEG-catalase, and NONOate were supplied by Sigma. H_2O_2, NaClO, KO_2, $NaNO_2$, $FeSO_4$ and other reagents were used as received from Shanghai Chemicals Ltd. Oil Red O and lactate dehydrogenase (LDH) assay kit were obtained from Nanjing Jiancheng Biology Engineering Institute. 4′, 6-diamidino-2-phenylindole (DAPI), paraformaldehyde, goat serum, Triton X-100 and mitochondrial membrane potential and apoptosis detection kit were purchased from Beyotime Biological Company. Pyroptosis marker protein caspase-1 probe (FAM-YVAD-FMK) was supplied by Bio-Rad Laboratories, Inc. Anti-NLRP3 inflammasome antibody, anti-IL-1β antibody, anti-IL-18 antibody, anti-caspase-1 antibody, and Alexa fluor-conjugated secondary antibody were obtained from Thermo Fisher Scientific. Dulbecco's modified Eagle's medium (DMEM, glucose 4.5 g/L, Gibco) and fetal bovine serum (FBS) were purchased from Gibco (Tulsa, OK, USA). Acetonitrile, methanol, ethanol, acetic acid, and dichloromethane were used after purification.

HClO was obtained by diluting aqueous NaClO in PBS buffer (pH 7.4). Hydroxyl radical ($^{\bullet}OH$) was generated in the Fenton system from ferrous sulfate and hydrogen peroxide (H_2O_2). Singlet oxygen ($^1O_2$) was produced from the HClO-H_2O_2 system in PBS buffer. Superoxide ($O_2^{\bullet-}$) was got by dissolving KO_2 in DMSO. Peroxynitrite ($ONOO^-$) was obtained by mixing 1 mM $NaNO_2$ and 1 mM H_2O_2. Nitric oxide (NO) originated from 1 mM NONOate. Hydrogen peroxide (H_2O_2) was diluted immediately from a stabilized 30% solution.

NMR spectra were performed on a Bruker 400 MHz Ultrashield Spectrometer. High-resolution mass spectra (HR-MS) were obtained using an LTQ Orbitrap XL hybrid FTMS (Fourier Transform Mass Spectrometer, Thermo Fisher Scientific). UV-visible absorption spectra were measured with a HITACHI UH5300 spectrometer. Fluorescent spectra were recorded on a HITACHI F-7000 fluorescence spectrophotometer. Fluorescent images were obtained on a laser scanning confocal microscope (Leica TCS SP8X).

2. QVB-B probe preparation

Synthesis of QVB: Terephthalaldehyde (1.35 g, 10.06 mmol) and 4-methylquinoline (4.32 g, 30.18 mmol) were dissolved in anhydrous ethanol, two drops piperidine and acetic acid were added and the solution was refluxed for 10 h. After cooling to room temperature, the

solution was concentrated under vacuum, and the product was purified through a silica gel column (eluent: PET/ CH_2Cl_2 = 50/1, V/V) to obtain compound 1. ^{1}H NMR (400 MHz, d_6-DMSO, δ): 8.91-8.90 (d, J = 4.0 Hz, 2H, -quinoline H), 8.60-8.58 (d, J = 8.0 Hz, 2H, -quinoline H), 8.22-8.18 (d, J = 12.0 Hz, 2H, -quinoline H), 8.06-8.04 (d, J = 8.0 Hz, 2H, -quinoline H), 7.95 (s, 4H, -ArH), 7.90-7.89 (d, J = 4.0 Hz, 2H, =CH-), 7.83-7.79 (m, 2H, -quinoline H), 7.71-7.69 (d, J = 8.0 Hz, 2H, =CH-), 7.67-7.65 (d, J = 8.0 Hz, 2H, -quinoline H). ^{13}C NMR (100 MHz, d_6-DMSO, δ) 150.19, 148.15, 142.06, 136.39, 134.56, 129.21, 127.98, 126.36, 125.55, 124.12, 122.44, 116.49. HR-MS (m/z, ESI): Cal. for [$C_{28}H_{20}N_2$], m/z=384.1626; [M+H] found, m/z=385.1689.

Synthesis of QVB-B: QVB (0.8 g, 2.08 mmol) and 4-(bromomethyl)-benzeneboronic acid pinacol ester (0.62 g, 2.08 mmol) were dissolved in acetonitrile. After the mixture and refluxed for 12 h, the solution was concentrated under vacuum, and the product was purified through a silica gel column (eluent: CH_2Cl_2/MeOH = 10/1, V/V) to obtain probe QVB-B as a bromine salt. ^{1}H NMR (400 MHz, d_6-DMSO, δ): 9.68-9.67 (d, J = 4.0 Hz, 1H, -quinoline H), 9.14-9.13 (d, J = 4.0 Hz, 1H, -quinoline H), 8.95-8.94 (d, J = 4.0 Hz, 1H, -quinoline H), 8.69-8.68 (d, J = 4.0 Hz, 1H, -quinoline H), 8.63-8.61 (d, J = 8.0 Hz, 1H, -quinoline H), 8.48-8.46 (d, J = 8.0 Hz, 1H, -quinoline H), 8.34-8.33 (d, J = 4.0 Hz, 1H, -quinoline H), 8.30-8.28 (d, J = 8.0 Hz, 1H, -quinoline H), 8.18-8.12 (m, 3H, -quinoline H and ArH), 8.08-8.00 (m, 5H, ArH and-CH=), 7.95-7.94 (d, J = 4.0 Hz, 1H, -quinoline H) 7.83-7.82 (d, J = 4.0 Hz, 1H, -quinoline H), 7.75-7.72 (d, J = 12.0 Hz, 2H, -ArH), 7.68-7.67 (d, J = 4.0 Hz, 2H, -CH=CH-), 7.37-7.36 (d, J = 4.0 Hz, 2H, -quinoline H and =CH-), 6.35 (s, 2H, -CH_2-), 1.26 (s, 12H,-CH_3). ^{13}C NMR (100 MHz, d_6-DMSO, δ) 153.72, 148.58, 143.36, 138.99, 138.09, 137.74, 135.95, 135.41, 135.18, 129.75, 129.68, 129.44, 128.38, 127.17, 126.89, 126.52, 125.90, 124.41, 124.08, 120.42, 119.66, 116.90, 83.95, 69.89, 59.43, 24.74. HR-MS (m/z, ESI): Cal. for [$C_{41}H_{38}BBrN_2O_2$]$^+$, m/z=652.2148; [M-Br]$^+$ found, m/z=601.3000.

3. Cell cultures

Human hepatoma cells (HepG2) were purchased from Shanghai Institute of Biochemistry and Cell Biology (Chinese Academy of Sciences, China). The cells were cultured in a Dulbecco's modified Eagle's medium (DMEM, glucose 4.5 g/L) supplemented with 10% fetal bovine serum (FBS), streptomycin at 100 mg/mL, penicillin at 100 U/mL, 4 mM *L*-glutamine at 37 °C in a 5% CO_2 /95% atmosphere.

4. Cytotoxicity assay

The cytotoxicity of QVB-B in Hep G2 cells was assessed using the MTT assay. Briefly,

cells (5, 000 cells/well) were seeded in 96-well plates. The plates were maintained at 37 °C in a 5% CO_2/95% air incubator for 24 h, followed by exposure to 200 μL of high glucose medium containing various concentrations of QVB-B (0, 2, 4, 6, 8, 10, 15, and 20 μM). After 12 h, the culture media were removed, and an MTT solution (20 μL, 5 mg/mL) and 180 μL of fresh medium were added to each well. After 4 h, the medium was removed, and 150 μL DMSO was added to dissolve the formazan crystals (10 min incubation in the dark). The absorbance at 570 nm for each well was measured on a microplate reader, and the cell viability was calculated.

5. Confocal imaging in live cells

HepG2 cells were seeded onto glass-bottomed dishes and cultured for 24 h at 37 °C in a humidified atmosphere. For monitoring of exogenous/endogenous H_2O_2 in live cells, the cells were treated with 10 μM QVB-B for 30 min in high glucose media at 37 °C and washed with PBS three times. After incubation with the corresponding concentration (0, 25, 50, and 100 μM) of H_2O_2 for 30 min or PMA (0, 1, 2, and 4 μg/mL) for 2 h, the cells were rinsed with PBS and imaged on a Leica laser scanning confocal microscope. The fluorescent signals were measured in the spectral range of 600–650 nm (red channel) and 420–470 nm (blue channel) using 405 nm excitation.

6. Ratiometry imaging H_2O_2 levels vary during pyroptosis

For ratio monitoring of endogenous H_2O_2 levels vary during palmitic acid (PA)-induced pyroptosis, the cells were treated with 0.2 mM PA for different time (0, 6, 12 and 24 h) or different concentrations (0, 0.05, 0.1, and 0.2 mM) for 24 h in high glucose, then, the cells were washed with PBS three times. After incubation with 10 μM QVB-B for 30 min, the cells were rinsed with PBS three times and imaged.

7. Revealing the correlation between H_2O_2 and pyroptosis

For revealing the correlation between H_2O_2 and pyroptosis, the cells were treated with 0.2 mM PA for 24 h in high glucose, then, the cells were washed with PBS three times. Subsequently, the cells were sequentially incubated with 10 μM QVB-B for 30 min and 2 μM FAM-YVAD-FMK (detection of pyroptosis-specific protein caspase-1) for 2 h, the cells were rinsed with PBS three times and imaged. The fluorescent signals of FAM-YVAD-FMK were measured in the spectral range of 500–550 nm using 490 nm excitation.

8. Oil Red O staining

HepG2 cells were incubated with different concentration PA (0, 0.05, 0.1 and 0.2 mM) for

24 h in high glucose media at 37 °C, then, the cells were washed with PBS three times. Subsequently, the cells were fixed with 10% formaldehyde for 30 min and stained with Oil Red-O for 10 min. After that, the cells were sequentially rinsed in 75% ethanol and 60% isopropanol to remove excess dyes. Lastly, the cells were sealed by glycerol gelatin and observed under the microscope.

9. Cell death assay

Pyroptotic cell death was evaluated with LDH release assay, Hoechst 33342/PI staining and annexin V-FITC and mitochondrial membrane potential staining. For LDH release, HepG2 cells were incubated without/with 0.2 mM PA for 24 h in high glucose. Then, the cell culture supernatants were collected and the LDH activity was detected using the LDH assay kit. Briefly, 25 μL cell supernatants and 25 μL substrate were mixed and incubated at 37 °C for 15 min. Then 25 μL 2, 4-dinitrophenylhydrazine was added into the samples and incubated at 37 °C for 15 min. Finally, 250 μL 0.4 mol/L NaOH solution was added and incubated at room temperature for 5 min. The absorbance was measured at 450 nm on a spectrophotometric microplate reader.

For Hoechst 33342/PI staining, HepG2 cells were incubated without/with 0.2 mM PA for 24 h in high glucose. Then, the cells were then incubated with a mixed solution of Hoechst 33342 and PI for 30 min and photographed under a fluorescence microscope.

For annexin V-FITC and mitochondrial membrane potential detection, briefly, the cells were incubated without/with 0.2 mM PA for 24 h and then washed with PBS. After that, 188 μL Annexin V-FITC binding solution, 5 μL Annexin V-FITC, 2 μL Mito-Tracker Red CMXRos and 5 μL Hoechst 33342 were mixed and incubated at room temperature in the dark for 30 min. Lastly, the samples were immediately observed under fluorescence microscope.

10. Immunofluorescence assay

Immunofluorescence staining was performed to detect the expression of NLRP3 inflammasome, caspase-1, mature IL-1β and IL-18 in HepG2 cells. The cells were incubated with 0.2 mM PA or 100 μM H_2O_2 for 24 h, followed by washing with PBS. Then, the cells were fixed with 4% paraformaldehyde for 30 min, penetrated by 0.5% Triton X-100 for 1 h, and blocked with goat serum. Subsequently, the cells were incubated with anti-NLRP3 inflammasome antibody, anti-caspase-1 antibody, anti-IL-1β antibody, or anti-IL-18 antibody at 4 °C overnight, followed by incubation with Alexa Fluor-conjugated secondary antibody in the dark for 1 h. The nuclei were stained by 4′, 6-diamidino-2-phenylindole (DAPI) for 20 min. The cells were imaged under Leica laser scanning confocal microscope.

Supplementary Figures

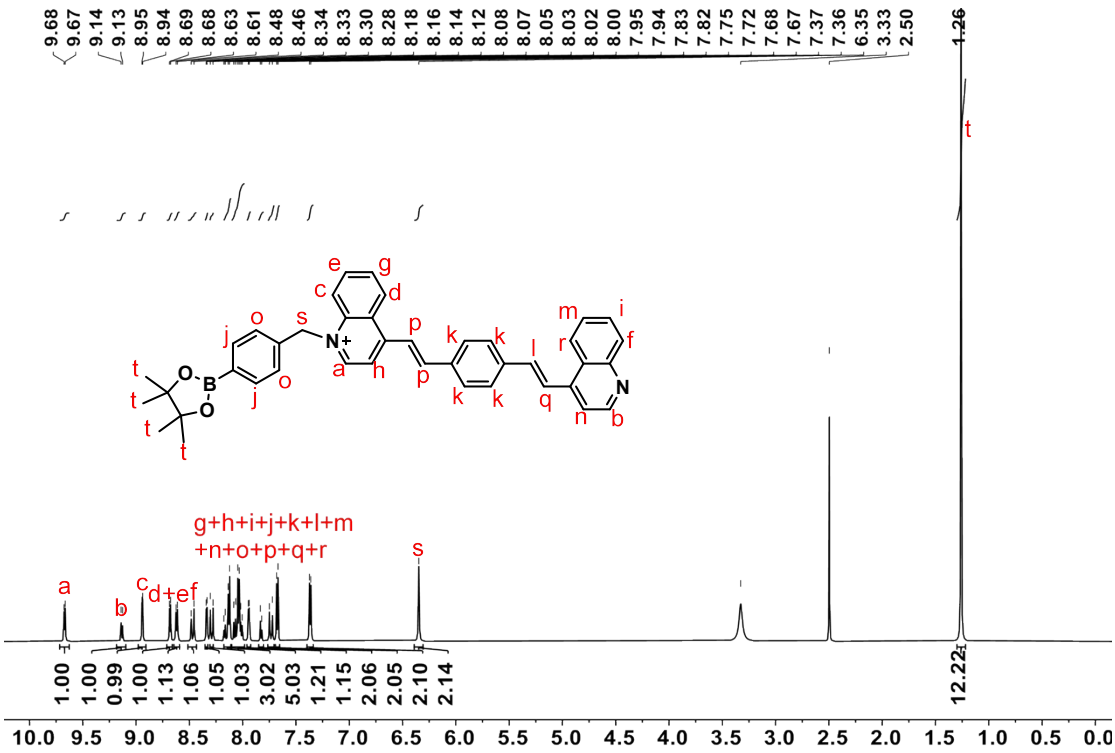

Figure S1. [1]H-NMR spectrum of QVB-B in d_6-DMSO.

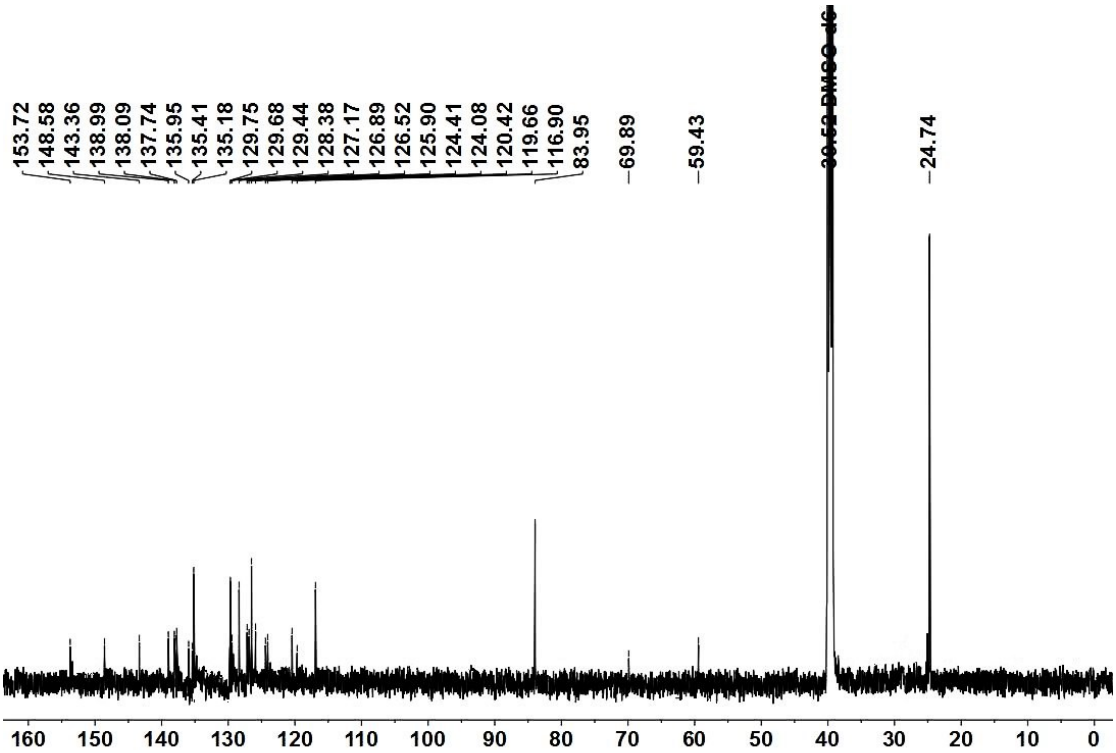

Figure S2. [13]C-NMR spectrum of QVB-B in d_6-DMSO.

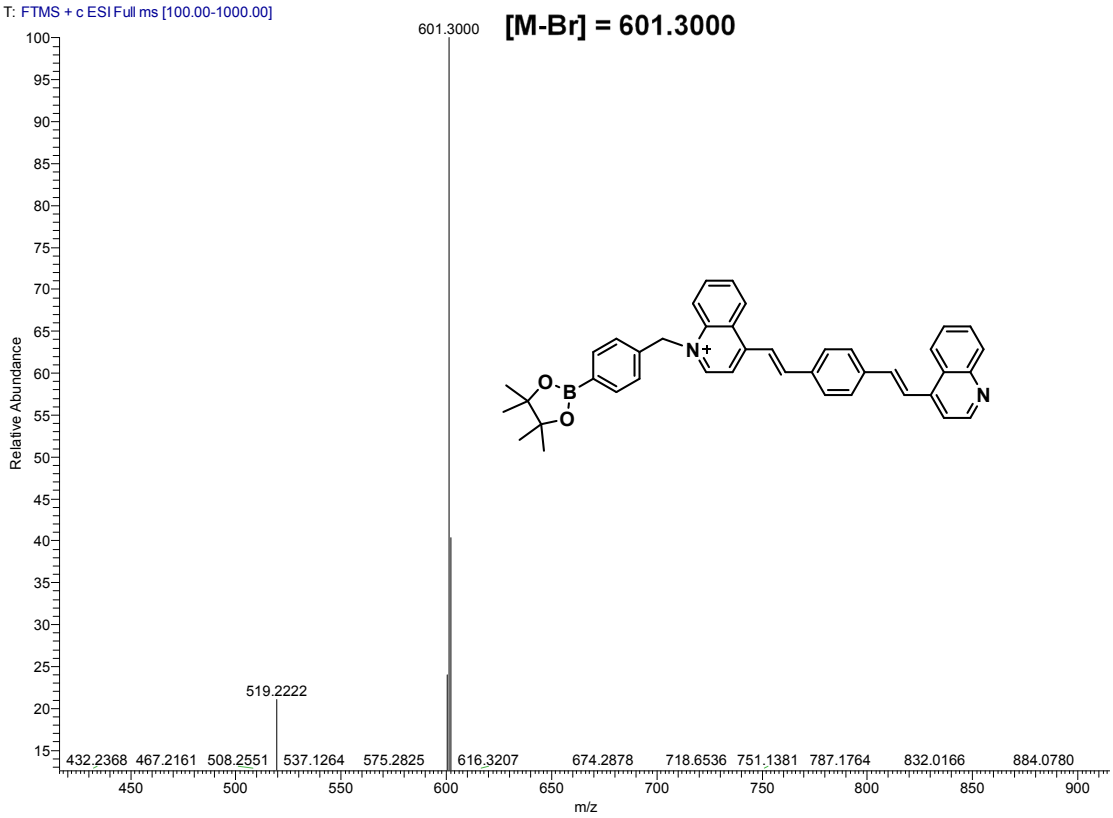

Figure S3. HR-MS spectrum of QVB-B ($[M-Br]^+ = 601.3021$).

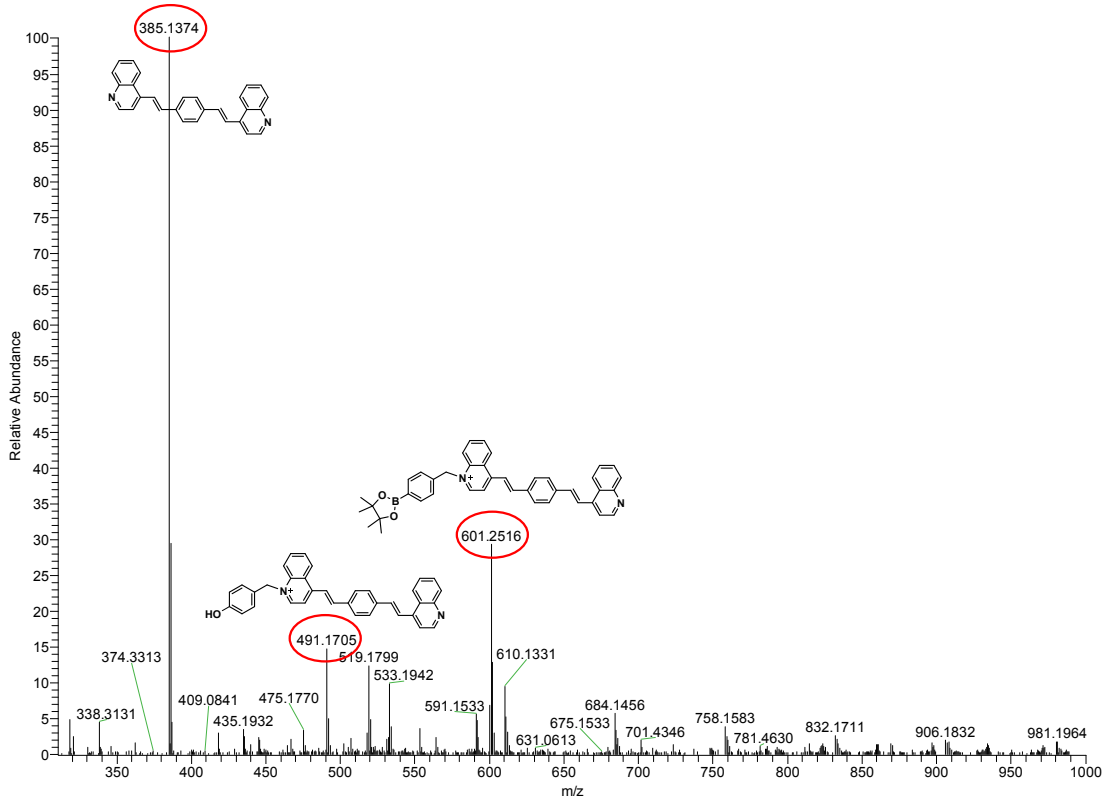

Figure S4. HR-MS spectrum of QVB-B switched on with H_2O_2.

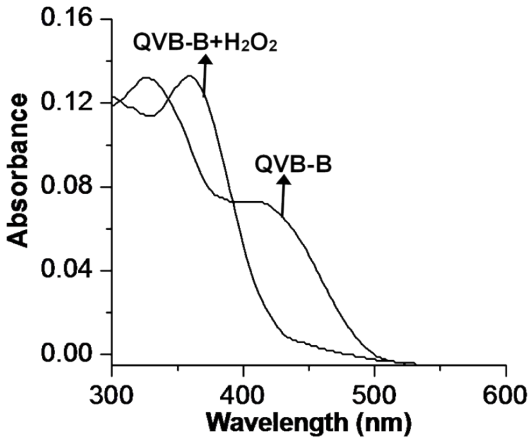

Figure S5. Characterization of QVB-B (10 μM) and QVB-B (10 μM) + H_2O_2 (100 μM) spectroscopic properties under physiological conditions.

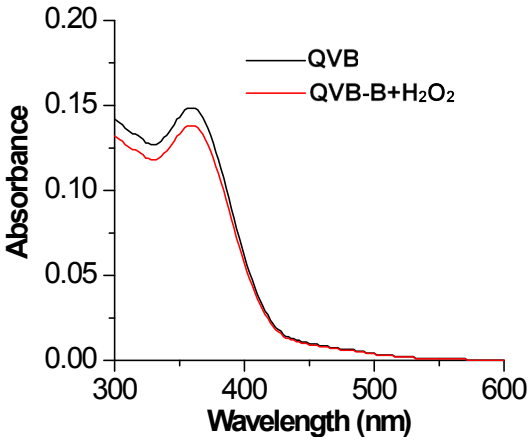

Figure S6. UV-vis absorption spectra of QVB (10 μM) and QVB-B (10 μM) + H_2O_2 (100 μM).

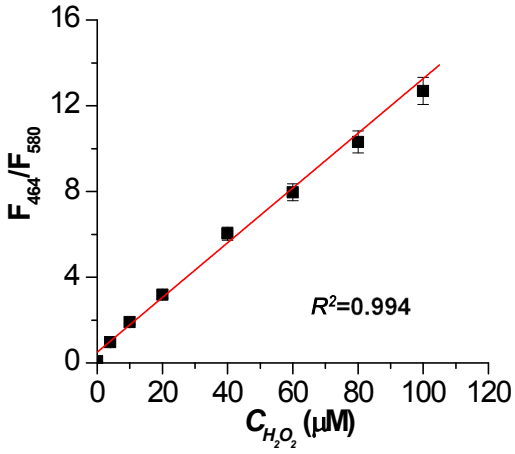

Figure S7. Concentration-dependent fluorescent ratios of the QVB-B probe switched on with H_2O_2. There is a good linear correlation in the range of 4-100 μM H_2O_2 (y = 0.38+0.13x, R^2=0.994).

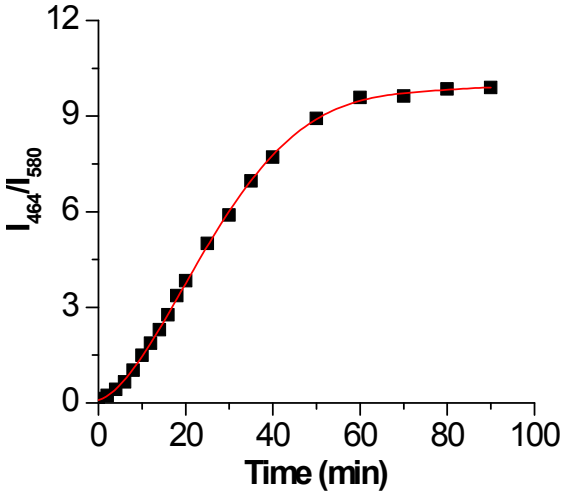

Figure S8. The time course of fluorescence intensity ratio (I_{464}/I_{580}) of probe after adding 100 μM H_2O_2.

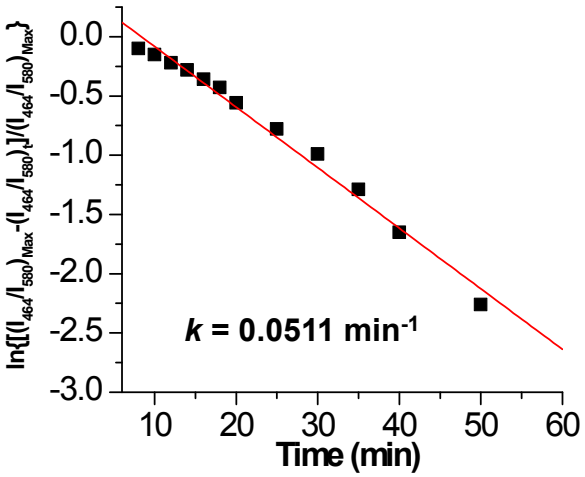

Figure S9. Pseudo-first-order kinetic plot of probe switched on with H_2O_2. The pseudo-first-order rate constant k for the reaction was calculated to be 0.0511 min^{-1} according to eqn: $\ln\{[(I_{464}/I_{580})_{Max}-(I_{464}/I_{580})_t]/(I_{464}/I_{580})_{Max}\} = -kt$.[1] Where $(I_{464}/I_{580})_t$ and $(I_{464}/I_{580})_{Max}$ are the fluorescence ratio at time t and the maximum ratio value obtained after the reaction was complete. k is the pseudo-first-order rate constant.

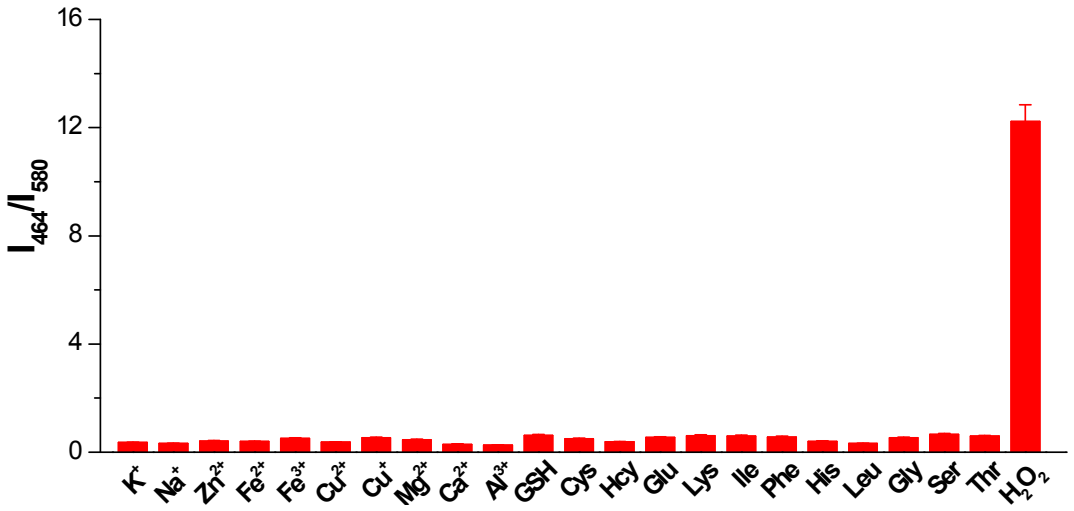

Figure S10. The selectivity of QVB-B (10 μM in PBS) responses to various biomolecules such as inorganic salts and amino acids.

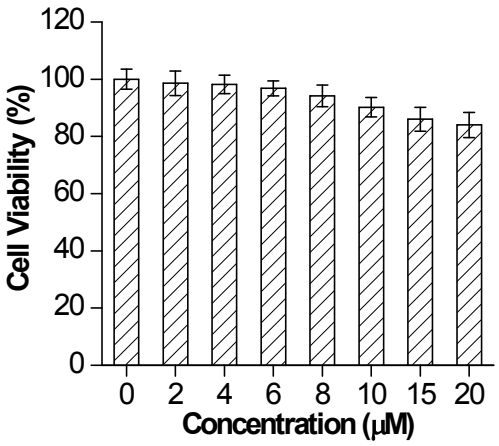

Figure S11. Viability of HepG2 cells in the presence of the QVB-B probe measured with the MTT assay.

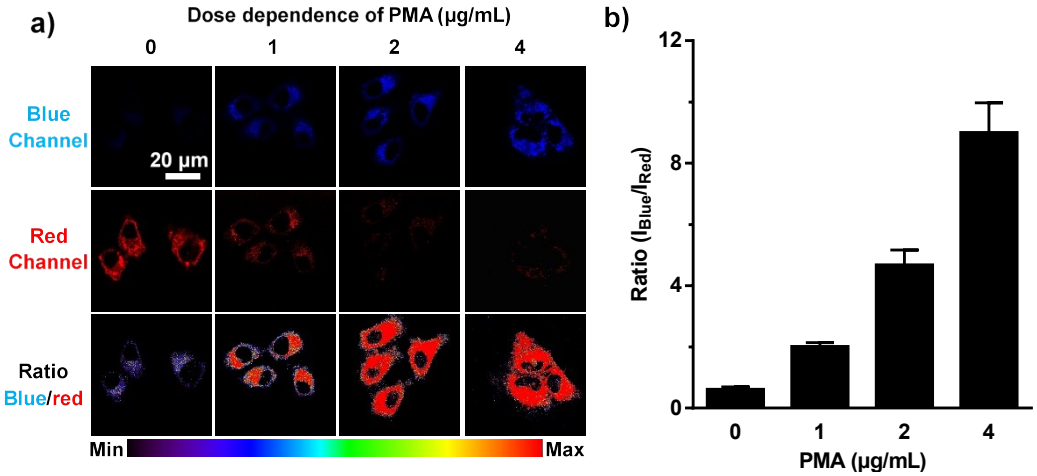

Figure S12. (a) Dose-dependent fluorescent ratiometric images after treatment with different concentrations of PMA. (b) Fluorescent ration under different treatment. The fluorescent ratios were obtained from six images with an identical observation area. The error bars represent the standard deviation (±SD).

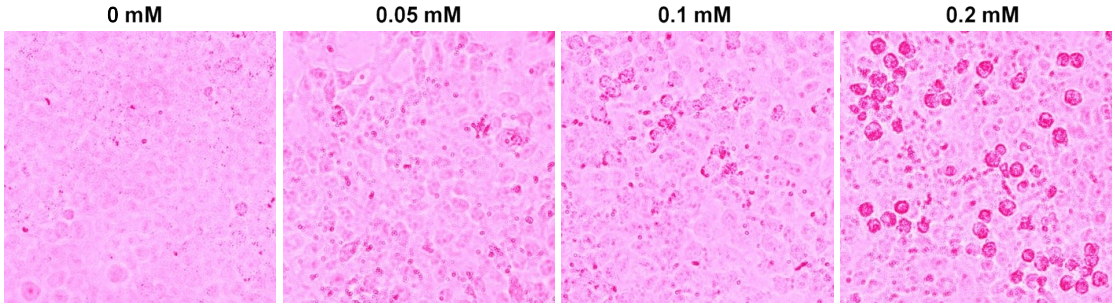

Figure S13. Identify the changes in intracellular PA deposition by the Oil Red O staining. The lipid accumulation levels were gradually increased with increasing PA concentration.

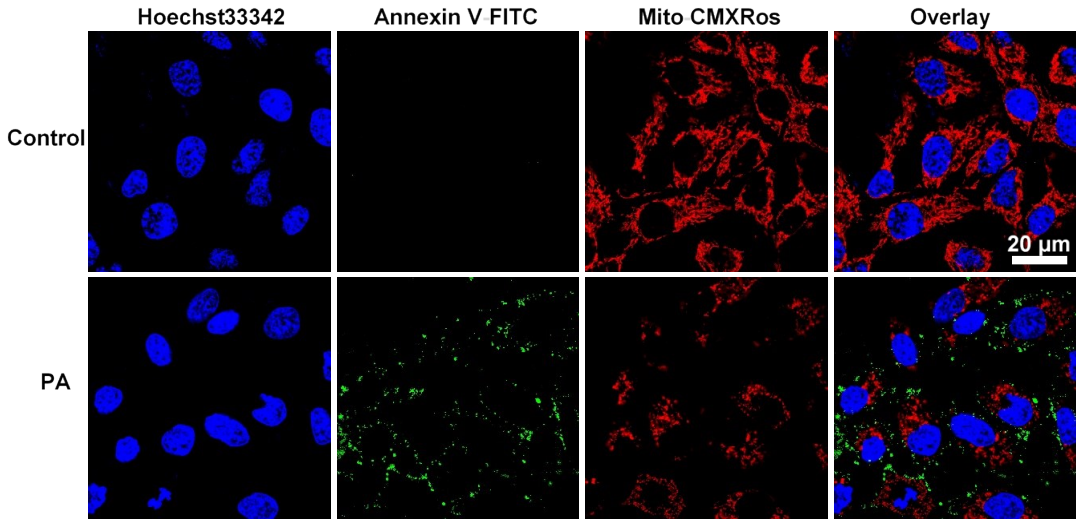

Figure S14. Cell death was determined by double staining of annexin V-FITC (green) and mitochondrial membrane potential (red).[2] After treatment with PA, the levels of annexin increased, and the mitochondrial membrane potential decreased.

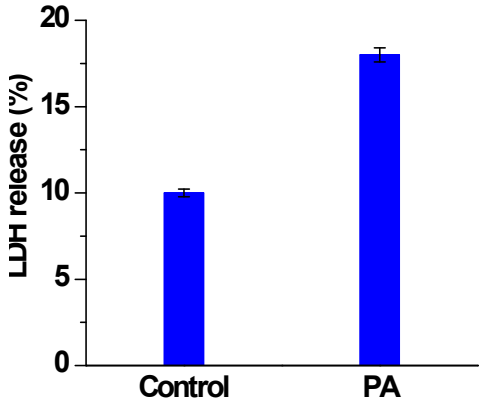

Figure S15. Pyroptosis cell death was conducted by LDH release assay. To discriminate between that apoptotic and pyroptotic cell death, we went on to conduct lactate dehydrogenase (LDH) release assay in PA-treated HepG2 cells. During pyroptosis, pores can be formed in the cell membrane and lead to the release of cellular contents and positive staining of dead cells, which can be determined by LDH release assay.[3] The error bars represent the standard deviation (±SD).

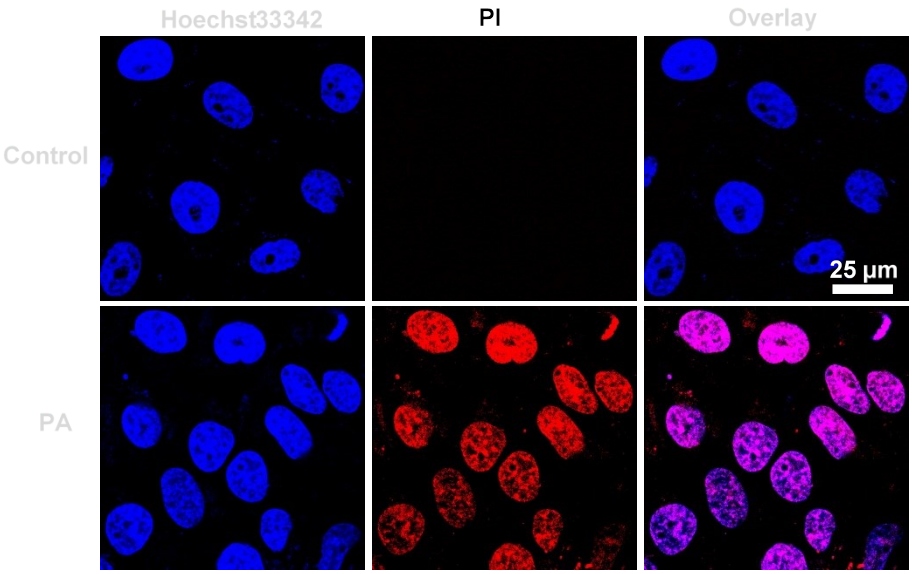

Figure S16. Pyroptosis cell death was conducted by PI staining. To further discriminate between that apoptotic and pyroptotic cell death, we also went on to PI-staining in PA-treated HepG2 cells. During pyroptosis, pores can be formed in the cell membrane and lead to positive staining of dead cells, which can be determined by PI staining.[4]

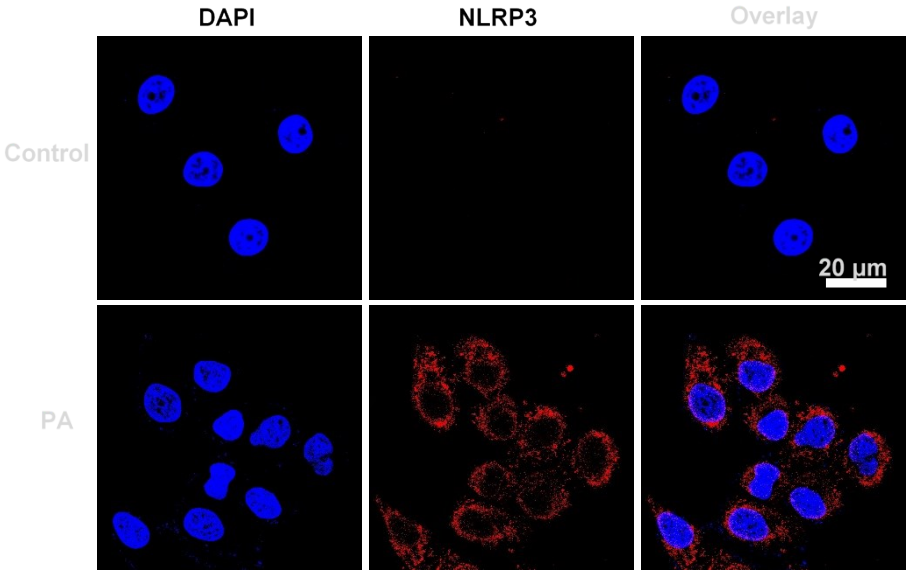

Figure S17. Comparison of the expression levels of the NLRP3 in HepG2 cells by the immunofluorescence assay. The expression levels of the NLRP3 were increased in HepG2 cells after treatment with PA.

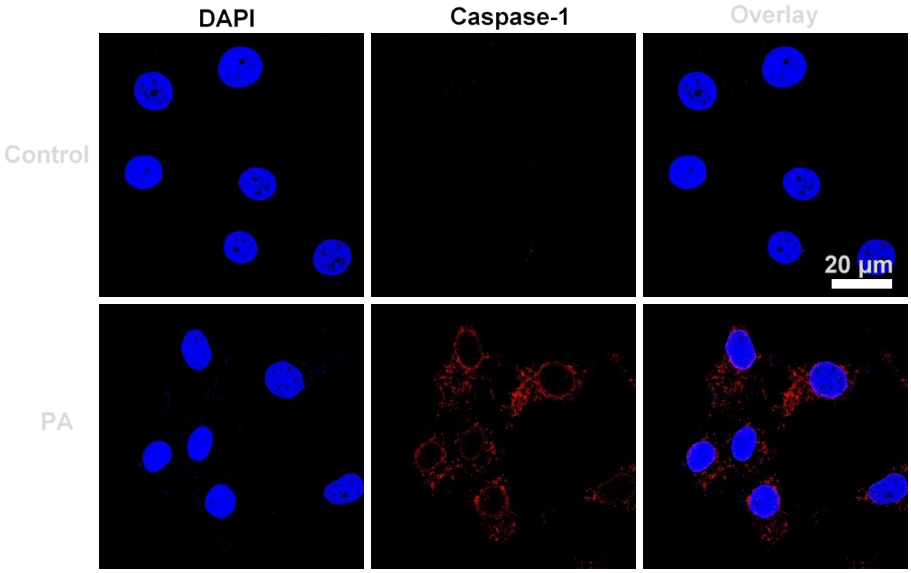

Figure S18. Comparison of the expression levels of the caspase-1 in HepG2 cells by the immunofluorescence assay. The expression levels of the caspase-1 were increased in HepG2 cells after treatment with PA.

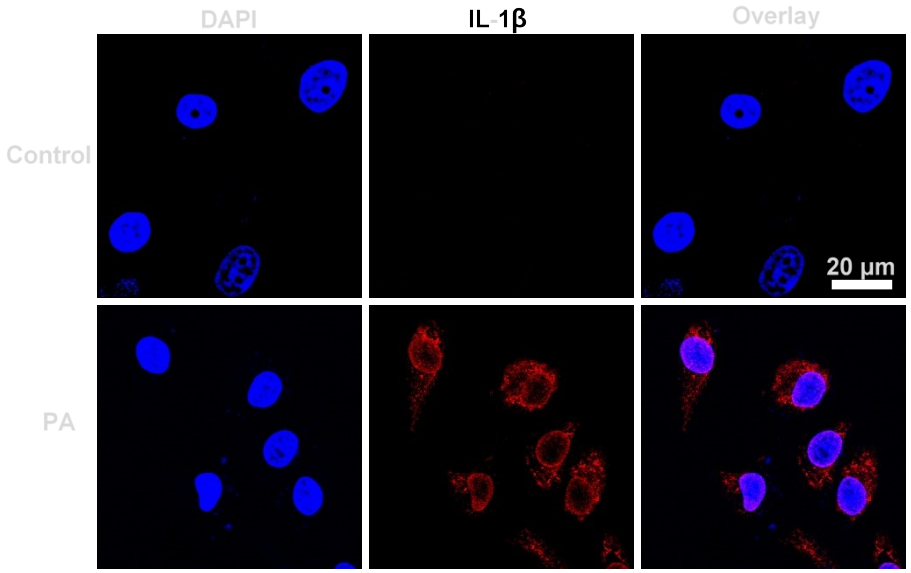

Figure S19. Comparison of the expression levels of the mature IL-1β in HepG2 cells by the immunofluorescence assay. The expression levels of mature IL-1β were increased in HepG2 cells after treatment with PA.

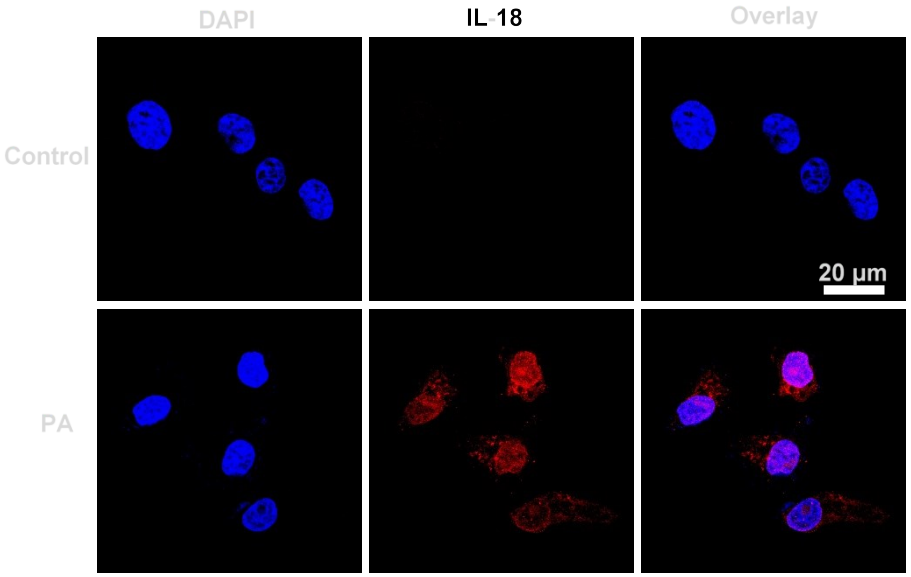

Figure S20. Comparison of the expression levels of the mature IL-18 in HepG2 cells by the immunofluorescence assay. The expression levels of mature IL-18 were increased in HepG2 cells after treatment with PA.

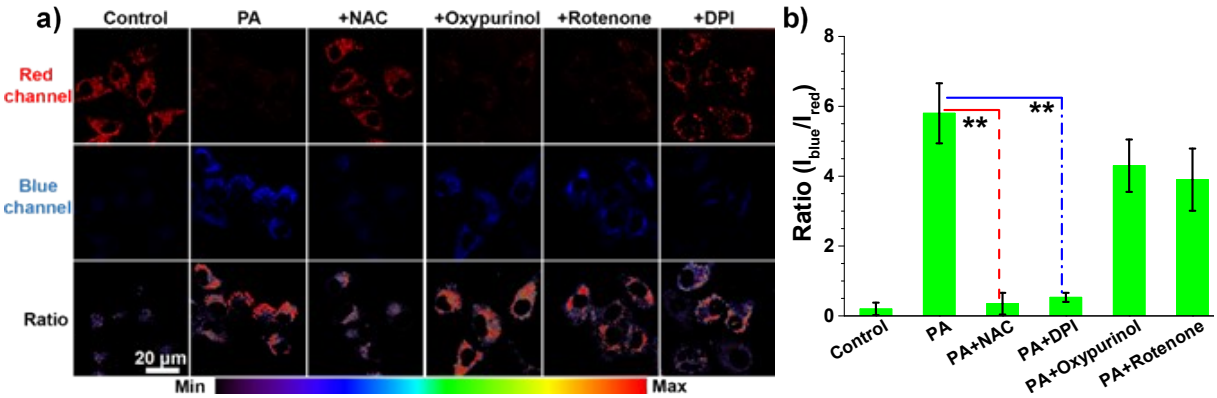

Figure S21. (a) Fluorescence ratio image of the main source of H_2O_2 induced by PA in HepG2 cells. Control: HepG2 cells without treatment PA; PA: HepG2 cells were treated with 0.20 mM PA for 24 h; +NAC: HepG2 cells were treated with NAC (H_2O_2 scavengers) for 4 h after treatment with PA; +Oxypurinol (Xanthine oxidase inhibitors): HepG2 cells were treated with 5 μm Oxypurinol and PA; +Rotenone (Mitochondrial respiratory chain enzyme complex inhibitors): HepG2 cells were treated with 5 μm Rotenone and PA; PA+DPI (NADPH oxidase complex inhibitors): HepG2 cells were treated with 5 μm DPI and PA. (b) Average fluorescence intensity ratios (blue/red) from images (A), **$p < 0.01$; as compared with PA.

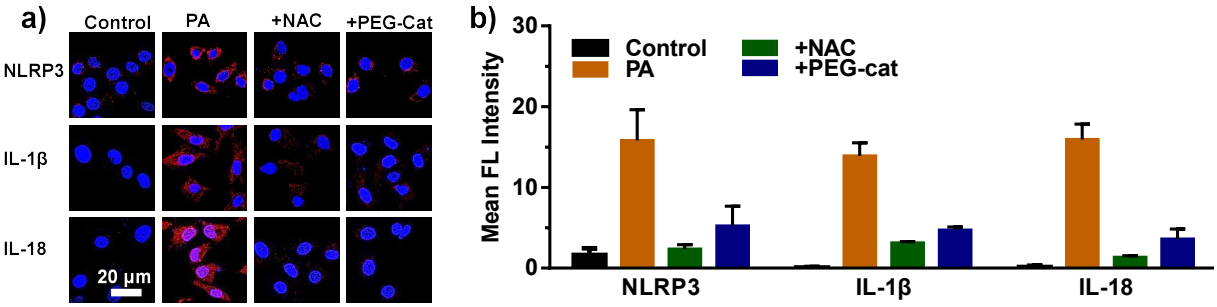

Figure S22. (a) Immunofluorescence signals from NLRP3 inflammasome, mature IL-1β and IL-18 under the PA induced the pyroptosis, and these signals were inhibited after specific eliminate H_2O_2. (d) Relative fluorescence intensities in a.

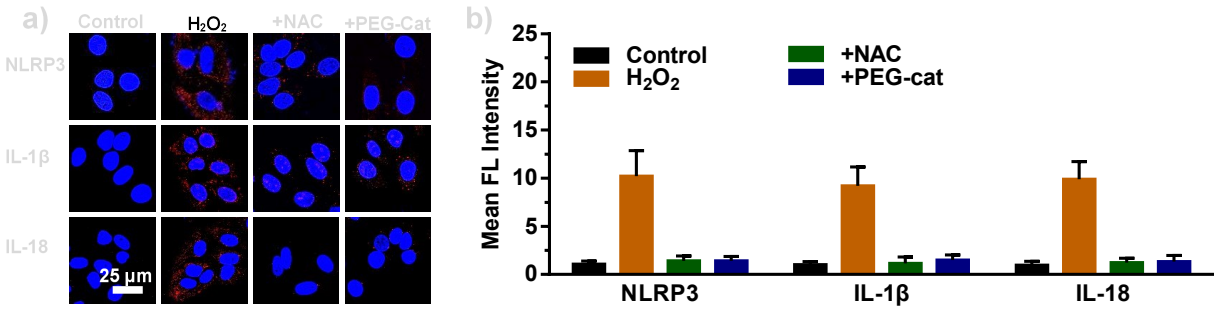

Figure S23. (a) Immunofluorescence signals from NLRP3 inflammasome, mature IL-1β and IL-18 under the treatment with exogenous H_2O_2, and these signals were inhibited after specific eliminate H_2O_2. (d) Relative fluorescence intensities in a.

References

(1) H. Xiao, P. Li, X. Hu, X. Shi, W. Zhang, B. Tang, B. *Chem. Sci.*, 2016, **7,** 6153–6159.

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