Supplementary Information

Selective detection of peroxynitrite using an isatin receptor and a naphthalimide fluorophore

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1. Experimental Details

1.1. General Experimental Information

Reagents and solvents were sourced from commercial suppliers and used directly as received. TLC was carried out on commercially available pre-coated aluminum-backed silica plates and compounds were visualized under UV light at 254 nm. Column chromatography was performed using 60 micron silica purchased from Sigma Aldrich. Melting points were recorded on a WRS-1 instrument and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded either in deuterated chloroform or dimethylsulfoxide at ambient temperature on a Bruker Avance 500 (500 MHz), with proton decoupling for all ^{13}C NMR spectra. HR-MS (high resolution mass spectra) was obtained on Agilent 6230, Thermo Fisher Scientific LTO FT Ultra, Thermo DFS Simm 210954 and HP-5989 instruments.

1.2. Synthesis of Compounds

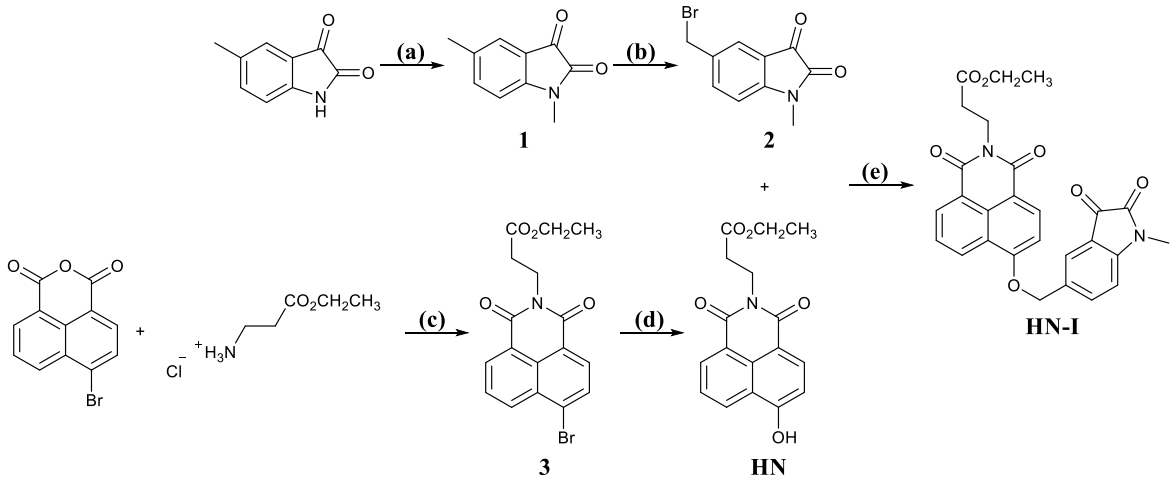
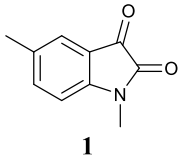


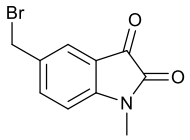
Figure S1. Synthesis of **HN** and **HN-I**. (a) K_2CO_3, MeI, MeCN, 70°C, 3h; (b) NBS, AIBN, CCl_4, reflux, 80°C, overnight; (c) DMAP, Et_3N, EtOH, reflux, 3h; (d) NHS, K_2CO_3, DMSO, 80°C, 100min; (e) KI, K_2CO_3, MeCN, 80°C, overnight.



1

1,5-Dimethylindoline-2,3-dione

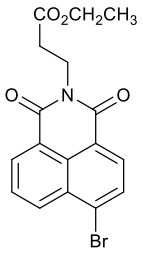
5-Methyl isatin (1.0 g, 6.2 mmol) and K_2CO_3 (1.72 g, 12.4 mmol) were dissolved in acetonitrile (15 ml), and then methyl iodide (1.32 g, 9.3 mmol) was added dropwise. The mixture was stirred at 70℃ for 3 h. After the reaction was complete, the solution was extracted with ethyl acetate (30 mL ×3). The organic layer was dried using anhydrous Na_2SO_4 and concentrated under reduced pressure. Finally, compound **1** (0.97 g, 90%) was obtained as a red solid.[1]



2

5-(Bromomethyl)-1-methylindoline-2,3-dione

Compound **1** (0.8 g, 4.6 mmol) and N-bromosuccinimide (NBS) (0.82 g, 4.6mmol) were dissolved in CCl_4 (30 mL), and the solution was heated at 80℃ and reflux for 10 minutes, 2,2'-azobis(2-methylpropionitrile) (AIBN) (0.08 g, 0.5 mmol) was added and the mixture refluxed overnight. Finally, the solvent was removed under reduced pressure, and the mixture was purified by column chromatography on silica gel (petroleum ether/ ethyl acetate = 4:1). Compound **2** (0.12 g, 10%) was obtained as a red solid.[1]

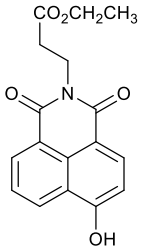


3

Ethyl 3-(6-bromo-1,3-dioxo-1H-benzo[de]isoquinolin-2(3H)-yl) propanoate

4-Bromo-1,8-naphthalic anhydride (0.5 g, 1.8 mmol) and β-alanine ethyl ester hydrochloride (0.35 g, 2.3 mmol) were dissolved in ethanol (20 mL) with the addition of 4-(dimethylamino) pyridine (0.13 g, 1.1 mmol) and triethylamine (0.3 mL). The mixture was stirred under reflux for 3 h. After the completion of the reaction, the solution was cooled to room temperature. Then, the mixture was filtered and washed by cold ethanol and water. Finally, compound **3** (0.52 g, 76%) was obtained as a white solid after column chromatography on silica gel (petroleum ether/ ethyl acetate = 4:1).[2]

^{1}H NMR (500 MHz, CH_3Cl): δ = 8.67 (dd, *J* = 6.5 Hz, *J* = 1.0 Hz, 1 H), 8.59 (dd, *J* = 8.5 Hz, *J* = 1.0 Hz, 1 H), 8.42 (d, *J* = 8.0 Hz, 1 H), 8.05 (d, *J* = 8.0 Hz, 1 H), 7.86 (dd, *J* = 8.5 Hz, *J* = 7.0 Hz, 1 H), 4.51-5.48 (m, 2 H), 4.17-4.12 (m, 2 H), 2.78-2.75 (m, 2 H), 1.24-1.21 ppm (m, 3 H); ^{13}C NMR (126 MHz, CH_3Cl): δ = 171.58, 163.82, 163.79, 133.81, 132.54, 131.71, 131.51, 131.05, 130.84, 129.40, 128.49, 123.30, 122.43, 61.10, 36.67, 33.10, 14.54 ppm; m.p.: 141-143 ℃; IR (KBr): υ = 1712 (s, C=O), 1663 (s, C=O), 1353 (s, C-O-C), 1205 (s, C-N); HRMS (ESI) calcd. for $C_{17}H_{14}BrNO_4$ $[M+H]^+$: 376.0184, found 376.0181.

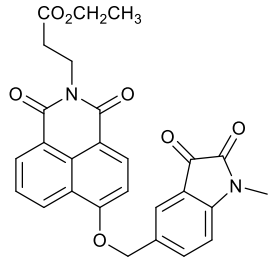


HN

Ethyl 3-(6-hydroxy-1,3-dioxo-1H-benzo[de]isoquinolin-2(3H)-yl) propanoate

Compound **3** (0.4 g, 1.0 mmol), N-hydroxysuccinimide (NHS) (0.13 g, 1.2 mmol) and K_2CO_3 (0.49 g, 3.5 mmol) were dissolved in DMSO (8 mL). The mixture was stirred at 80 ℃ for 100 minutes. After the completion of the reaction, the solution was cooled to room temperature, diluted with H_2O and acidified to pH = 1 with 1 M HCl. The precipitate was collected by vacuum filtration and washed with H_2O. Finally, compound **HN** (0.24 g, 75%) was obtained as a yellow solid.[3]

^{1}H NMR (500 MHz, CH_3Cl): δ = 8.52 (dd, *J* = 6.0 Hz, *J* = 1.0 Hz, 1 H), 8.33 (s, 1 H), 8.21 (dd, *J* = 8.0 Hz, *J* = 1.0 Hz, 1 H), 8.10 (d, *J* = 8.0 Hz, 1 H), 7.51 (dd, *J* = 8.5 Hz, *J* = 7.5 Hz, 1 H), 6.73 (d, *J* = 8.0 Hz, 1 H), 4.52 (t, *J* = 6.5 Hz,2 H), 4.29-4.25 (m, 2 H), 2.81 (t, *J* = 6.5 Hz,2 H), 1.36 ppm (t, *J* = 7.0 Hz, 3 H); ^{13}C NMR (126 MHz, DMSO): δ = 170.88, 163.55, 162.84, 160.36, 133.58, 131.12, 129.17, 128.97, 125.55, 122.35, 121.66, 112.42, 109.95, 60.07, 35.47, 32.36, 13.93 ppm; m.p.: 190-192 ℃; IR (KBr): υ = 3333 (s, O-H), 1700 (s, C=O), 1656 (s, C=O), 1359 (s, C-O-C), 1256 (s, C-N); HRMS (ESI) calcd. for $C_{17}H_{15}NO_5$ $[M+H]^+$: 314.1028, found 314.1025.



HN-I

Ethyl 3-(6-((1-methyl-2,3-dioxoindolin-5-yl) methoxy)-1,3-dioxo-1H-benzo[de]isoquinolin-2(3H)-yl) propanoate

Compound **4** (0.14 g, 0.5 mmol), compound **2** (0.12 g, 0.5 mmol), KI (6.1 mg, 0.08 equiv.) and K_2CO_3 (0.06 g, 0.5 mmol) were dissolved in acetonitrile (10 mL). The solution was refluxed at 80 ℃ overnight. After the completion of the reaction, the mixture was cooled to room temperature, diluted with 1 M HCl, extracted with DCM (3 × 10 mL), washed by brine (3 × 20 mL), dried over by anhydrous Na_2SO_4, filtered and evaporated in vacuo. Finally, compound **HN-I** (40 mg, 18%) was obtained as an orange solid by column chromatography on silica gel (dichloromethane / ethyl acetate = 4:1).[4]

^{1}H NMR (500 MHz, CH_3Cl): δ = 8.63 (dd, *J* = 7.0 Hz, *J* = 1.0 Hz, 1 H), 8.58-8.55 (m, 2 H), 7.79-7.77 (m, 2 H), 7.72 (dd, *J* = 8.5 Hz, *J* = 7.5 Hz, 1 H), 7.11 (d, *J* = 8.5 Hz, 1 H), 6.99 (d, *J* = 8.0 Hz, 1 H), 5.34 (s, 2 H), 4.50-4.47 (m, 2 H), 4.17-4.13 (m, 2 H), 3.30 (s, 3 H), 2.76 (t, *J* = 7.5 Hz, 2 H), 1.23 ppm (t, *J* = 7.0 Hz, 3 H); ^{13}C NMR (126 MHz, $CDCl_3$): δ = 183.08, 171.48, 164.29, 163.66, 159.41, 158.22, 151.72, 137.39, 133.39, 131.94, 131.57, 129.58, 128.75, 126.40, 124.72, 123.64, 122.43, 117.89, 115.67, 110.44, 106.49, 69.90, 60.77, 36.17, 32.94, 26.54, 14.28 ppm; m.p.: 212-214 ℃; IR (KBr): υ = 1733 (s, C=O), 1654 (s, C=O), 1354 (s, C-O-C), 1262 (s, C-N); HRMS (ESI) calcd. for $C_{27}H_{22}N_2O_7$ $[M+H]^+$: 487.1505, found 487.1506.

Preparation of DSPE-PEG/HN-I

Compound **HN-I** was dissolved in DMSO to prepare a 1 mM stock solution. DSPE-PEG2000 was dissolved in phosphate-buffered saline (PBS, pH = 7.4) for the preparation of a 1mM stock solution. **DSPE-PEG/HN-I** solution (**HN-1:** DSPE-PEG = 1:1) was prepared by mixing **HN-I** and DSPE-PEG using the two stock solutions. After incubating for 30min a **DSPE-PEG/HN-I** solution was obtained.

1.3. The Proposed Reaction Mechanism

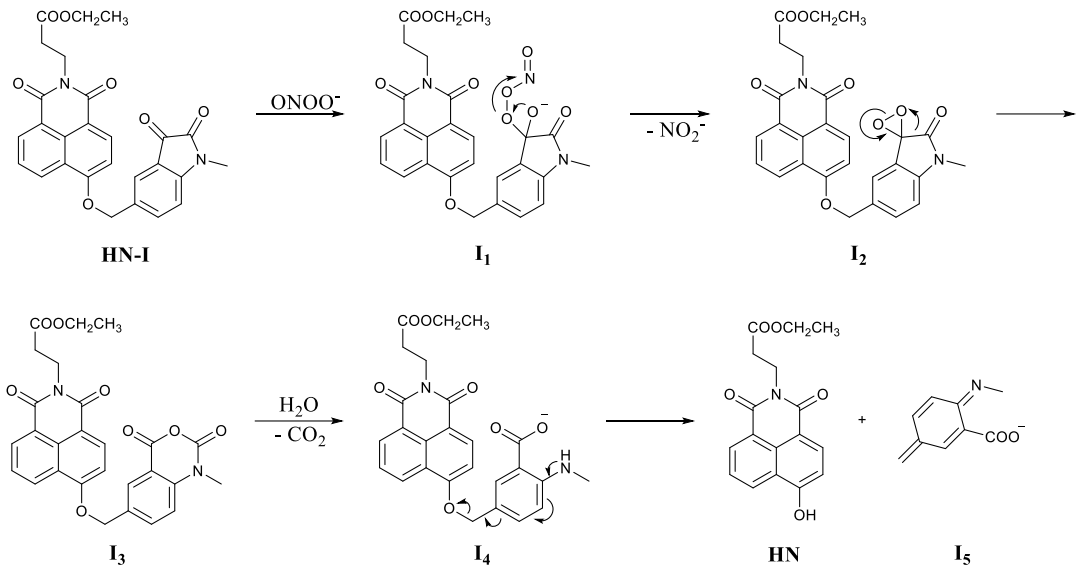


Figure S2. The proposed reaction mechanism between **HN-I** and $ONOO^{-}$.[1, 5]

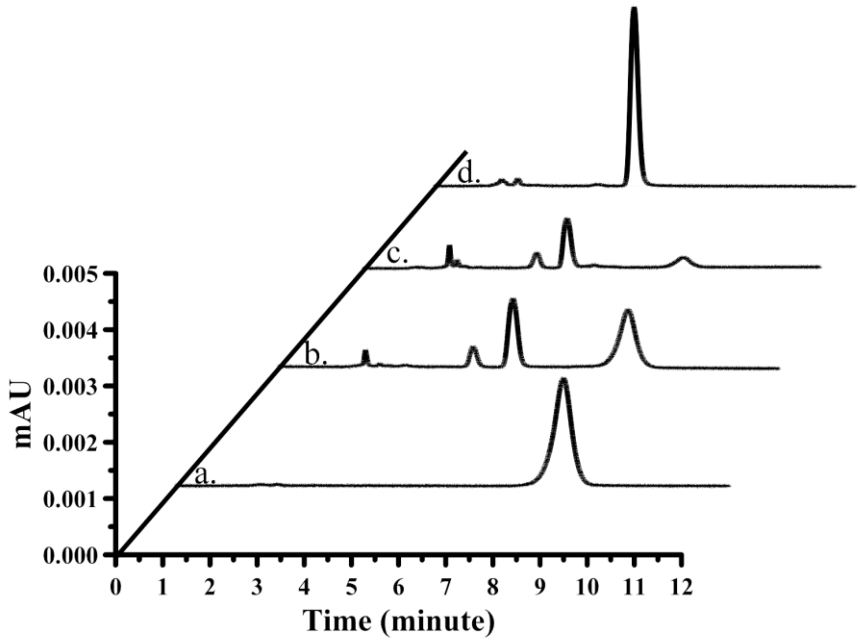


Figure S3. HPLC of 10 μM **HN-I** (a.), 10 μM **HN-I** with 5μM $ONOO^{-}$ (b.), 10 μM **HN-I** with 30 μM $ONOO^{-}$ (c.) and 10 μM HN in Methanol.

2. Fluorescence Measurements

Fluorescence measurements were performed on a RF-5301PC Fluorescence Spectrophotometer (Shimadzu, Japan). All pH measurements taken during fluorescence/absorption experiments were recorded on a pH meter (Shanghai Leici instrument factory). UV-VIS measurements were performed on a UV-2501PC Visible Spectrophotometer (Shimadzu, Japan).

Phosphate buffered saline (PBS) was freshly prepared from 52% methanol in water with KCl (10 mM), KH_2PO_4 (2.752 mM) and Na_2HPO_4 (2.757 mM). The PBS buffer was adjusted to pH 8.3 with 1 M HCl (aq).

Phosphate buffered saline (PBS) was freshly prepared from 100% water with KCl (10 mM), KH_2PO_4 (2.752 mM) and Na_2HPO_4 (2.757 mM). The PBS buffer was adjusted to pH 7.3 with 1 M HCl (aq).

Hydrogen peroxide (H_2O_2) is commercially available whereby the concentration of H_2O_2 was determined through spectrophotometrical analysis with $\varepsilon = 43.6\ cm^{-1}\ M^{-1}$ at 240 nm.

Peroxynitrite ($ONOO^-$) stock solutions were freshly prepared each time prior to usage. A solution of 3 M NaOH was cooled to 0 ℃ to which simultaneously 0.7 M H_2O_2, 0.6 m $NaNO_2$ and 0.6 M HCl were added. The $ONOO^-$ solution was analyzed spectrophotometrically whereby the concentration of $ONOO^-$ was estimated through $\varepsilon = 1670 \pm 50\ cm^{-1}\ M^{-1}$ at 302 nm in 0.1 M NaOH (aq.).

ROO• was generated from 2, 2'-azobis (2-amidinopropane) dihydrochloride. AAPH (2, 2‘azobis (2-amidinopropane) dihydrochloride, 0.1 M) was added into deionizer water, and then stirred at 37 °C for 30 min.

Superoxide was generated from KO_2. KO_2 (1.0 eq) and 18-crown-6 ether (2.5 eq) was dissolved in DMSO to afford a superoxide stock solution.

Hydroxyl radical was generated by the Fenton reaction. To prepare •OH solution, hydrogen peroxide (H_2O_2, 10 eq) was added to $Fe(ClO_4)_2$ in deionised water.

The concentration of ClO^- was determined from the absorption at 292 nm ($\varepsilon = 350\ M^{-1}\ cm^{-1}$).

Hydrogen Sulphide is directly obtained from sodium sulfide which is commercially available.

3. UV and Fluorescence Analysis

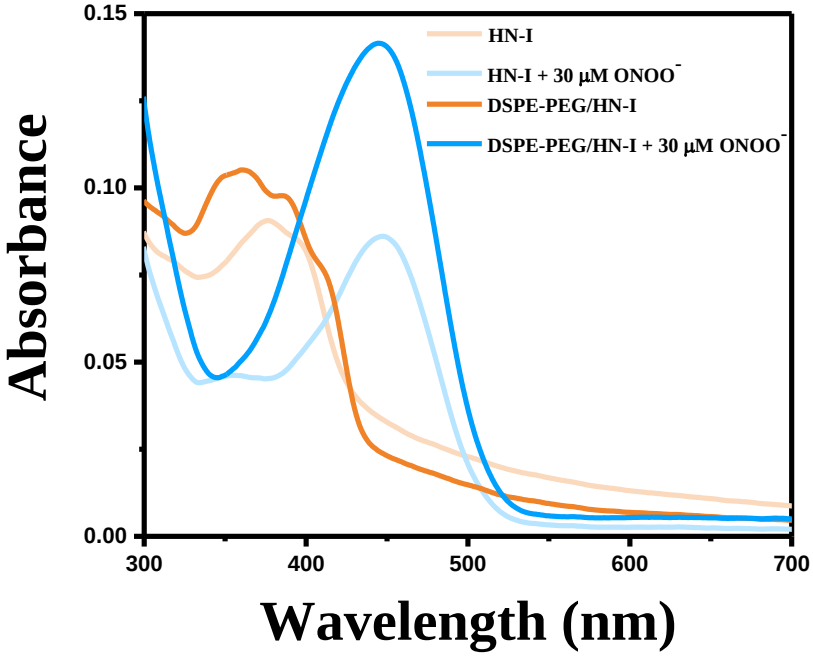


Figure S4. UV-VIS of **HN-I** (10 μM) or **DSPE-PEG/HN-I** (10/10 μM) with or without the addition of $ONOO^-$ (30 μM). The data was obtained in PBS buffer (5.5mM, containing 1% DMSO), pH = 7.4 at 25 ℃, λ_{ex} = 448 nm, λ_{em} = 563 nm.

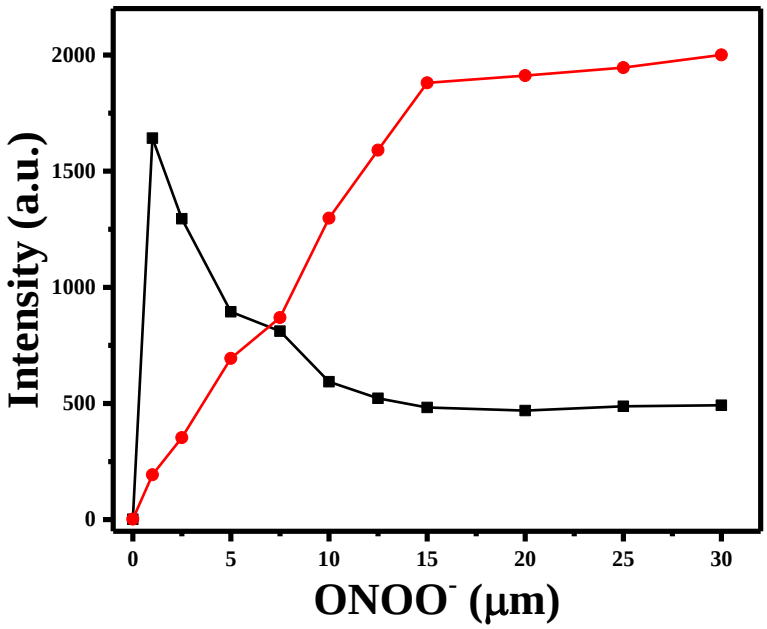


Figure S5. Dose dependent curves for **HN-I** (10 μM) in black and DSPE-PEG/HN-I(10 μM) in red upon the presence of $ONOO^-$ (0-30 μM). The data was obtained in PBS buffer (5.5mM, containing 1% DMSO), pH = 7.4 at 25 ℃, λ_{ex} = 448 nm, λ_{em} = 563 nm.

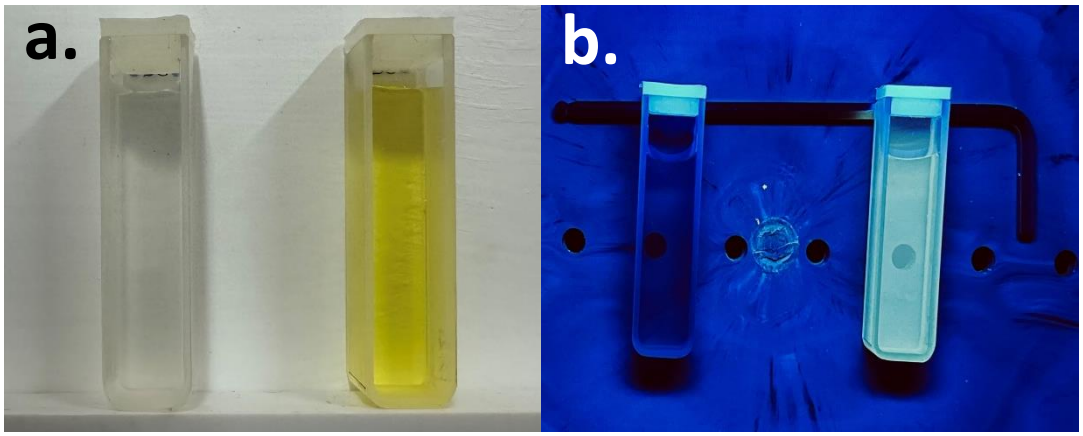


Figure S6. DSPE-PEG/HN-I without (left) and with (right) $ONOO^-$ under room light (a.) and UV light (b.).

4. Cytotoxicity Assays of DSPE-PEG/HN-I in Live HepG2 Cells

The cytotoxicity was assessed by a cell counting kit-8 (CCK-8) assay. Briefly, HepG2 cells were cultured in 96-well microplates incubator overnight at 37 °C in a humidified atmosphere of 5 % CO_2 and 95 % air. Then, cells were incubated with various concentrations (0/0, 5/5, 10/10, 20/20 and 40/40 μM) of **DSPE-PEG/HN-I** for 48 hours. Finally, CCK-8 solution (10 μL) was added into each well for 2 h, and absorbance at 450 nm was measured.

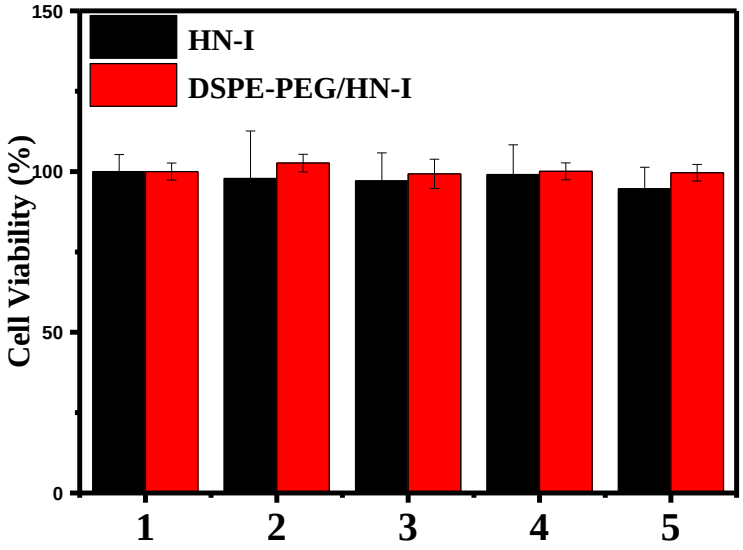


Figure S7. Cytotoxicity of live HepG2 cells incubated with different concentrations of **HN-I** (0, 5, 10, 20, 40 μM) or **DSPE-PEG/HN-I** (0/0, 5/5, 10/10, 20/20, 40/40 μM). DMSO was used at a concentration of 0.2%.

5. Cell Culture and Imaging

HepG2 (ATCC® HB-8065™), HeLa (ATCC-CCL2) and RAW 264.7 macrophages (ATCC® TIB-71™) were maintained in a Dulbecco's Modified Eagle's Medium (DMEM, Gibco,12800082) supplemented with 10 % FBS (Gibco, 2025790) in a humidified atmosphere of 5 % CO_2 and 95 % air at 37 °C and split when the cells reached 90 % confluency.

Cells were seeded on a black 96-well microplate with optically clear bottom (Greiner bio-one, Germany) overnight at 37 °C in a humidified atmosphere of 5 % CO_2 and 95 % air. First, cells were incubated with an $ONOO^-$ donor (SIN-1) to evaluate sensitivity to exogenous $ONOO^-$. Cells were incubated with **DSPE-PEG/HN-I** (20/20 μM) for 1 h, followed by incubation with SIN-1 for 4 h at 37 °C. To confirm the detection of exogenously generated $ONOO^-$, the cells were pre-incubated with N-acetylcysteine

(NAC, 1mM). To generate endogenous $ONOO^-$, LPS (1.0 μg/mL) was first incubated for 24 h in RAW 264.7 macrophages at 37 °C. In addition, one group of cells was pretreated with NAC (1 mM) and then incubated with LPS. After washing with PBS twice, the cells were incubated with **DSPE-PEG/HN-I** (20/20 μM) for 1 h at 37 °C. The cells were then washed three times with PBS. The fluorescence images were recorded using an Opera Phenix High Content Screening System (Perkinelmer, US) and quantified and plotted by columbus analysis system (Perkinelmer, US).

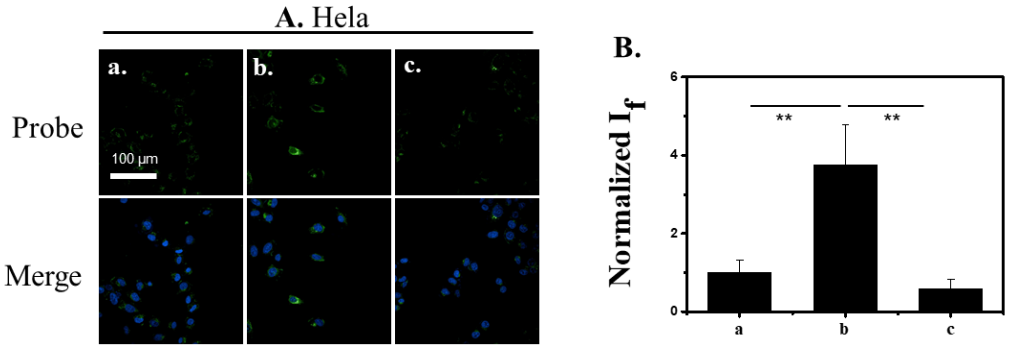


Figure S8. A) Imaging of exogenous $ONOO^-$ in HeLa cells. b) **DSPE-PEG/HN-I** (20/20 μM, 1 h)-loaded HeLa cells incubated with 1mM SIN-1 for 4 h, and then imaged. c) **DSPE-PEG/HN-I** (20/20 μM, 1 h)-loaded HeLa cells incubated with 1mM SIN-1 for 4 h in the presence of 1mM NAC, and then imaged. Normalized intensities in a-c (**B**). λ_{ex} = 488 nm, λ_{em} = 500-550 nm. DMSO was used at the concentration of 0.2%.

6. NMR Spectra

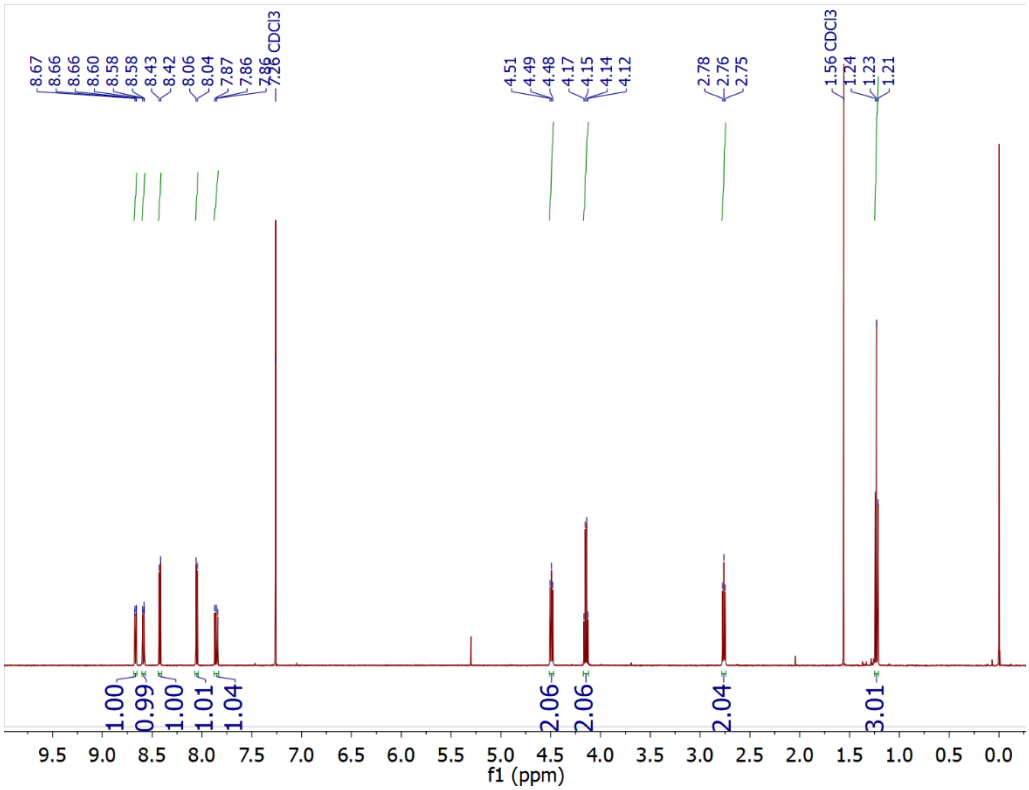


Figure S9. The ^{1}H NMR spectrum of compound **3**.

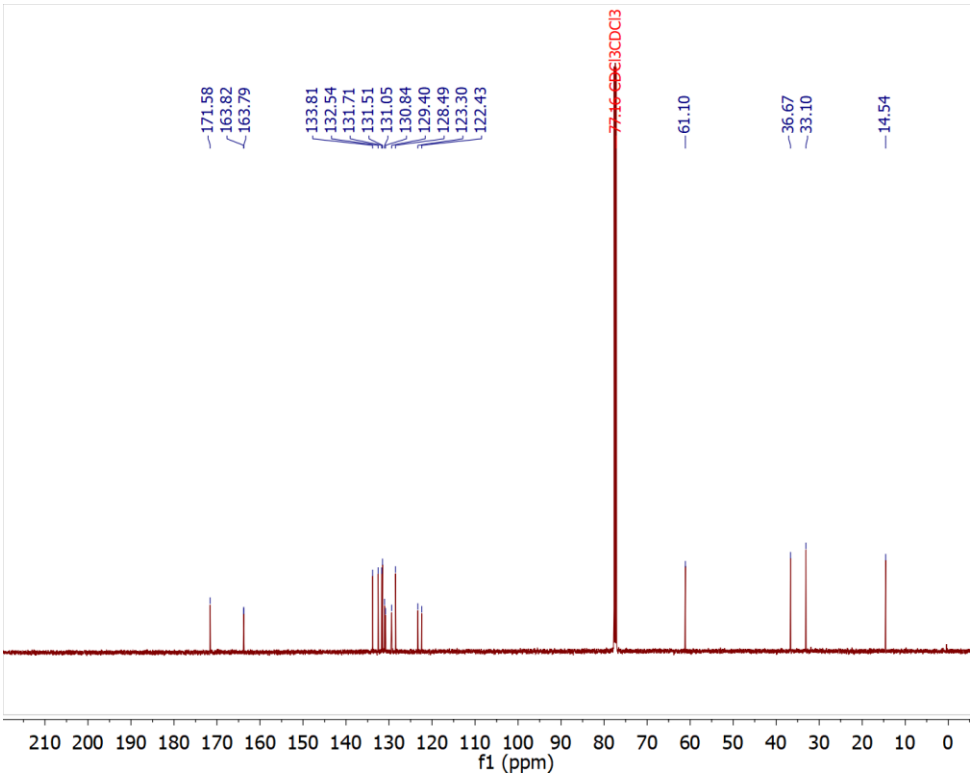


Figure S10. The ^{13}C NMR spectrum of compound **3**.

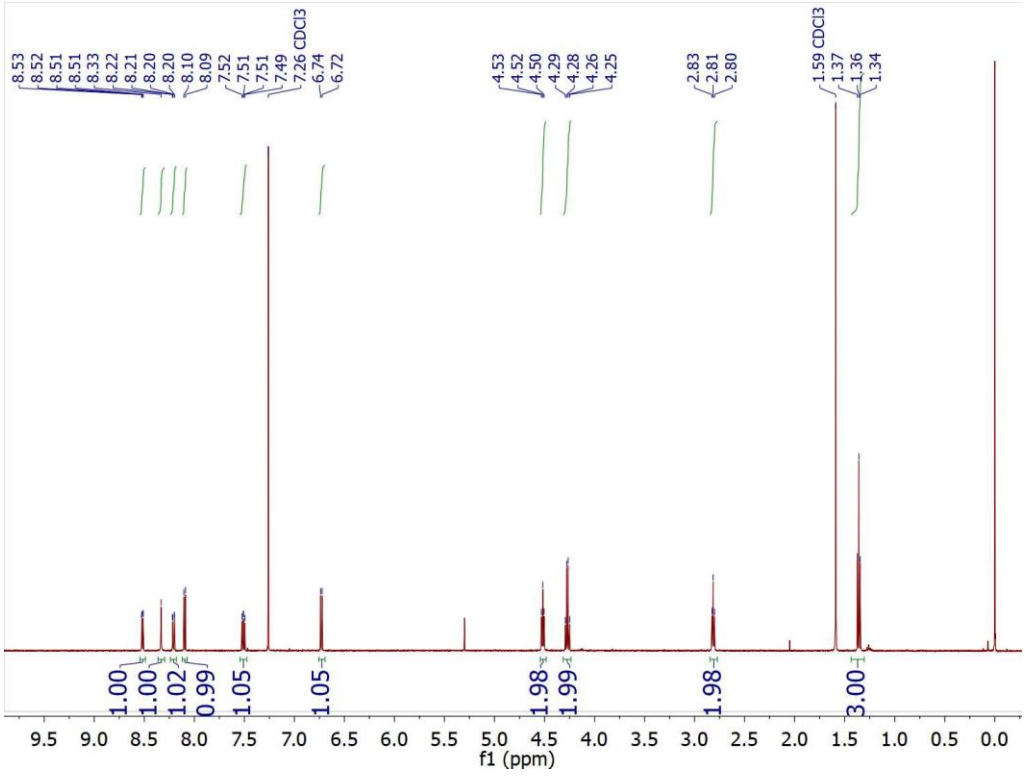


Figure S11. The ^{1}H NMR spectrum of **HN**.

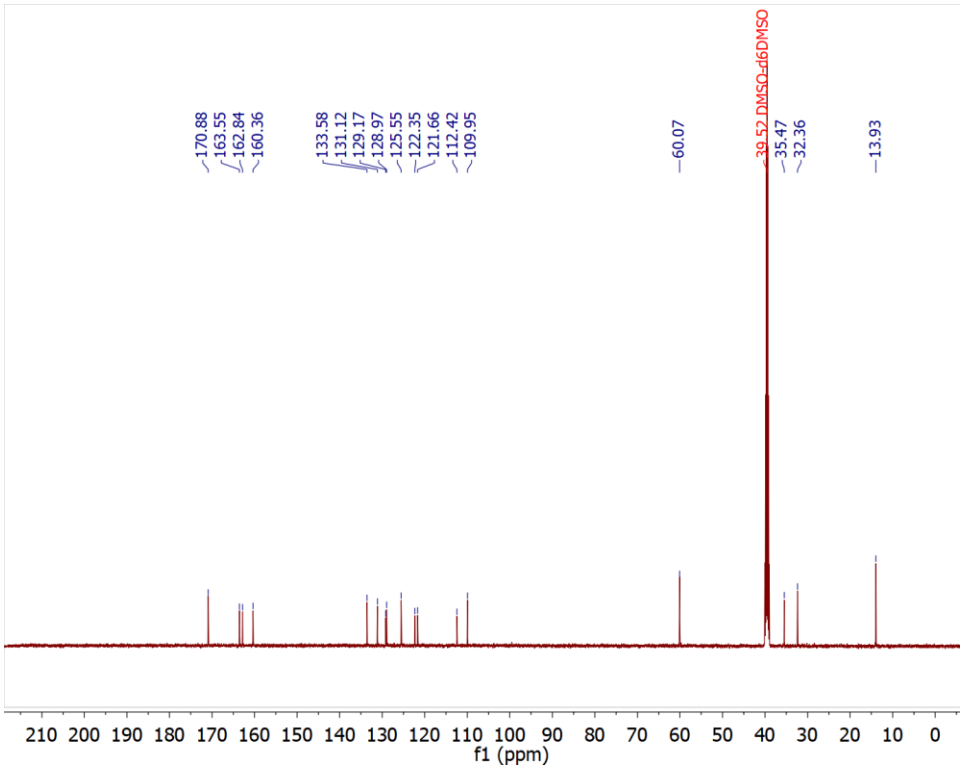


Figure S12. The ^{13}C NMR spectrum of **HN**.

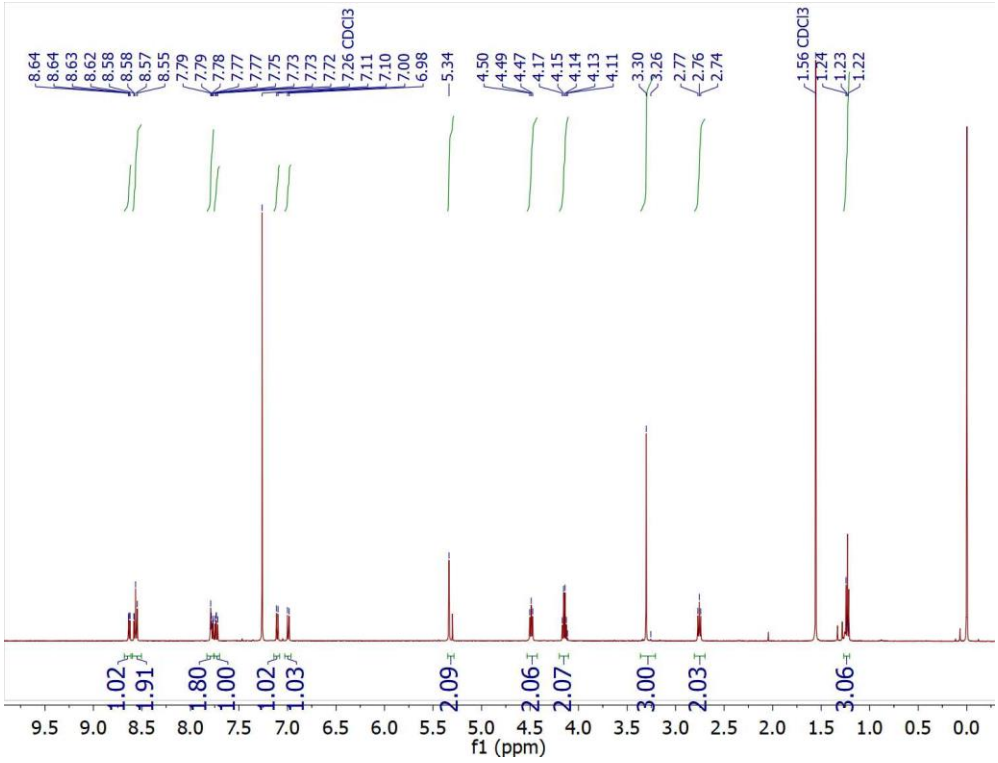


Figure S13. The ^{1}H NMR spectrum of **HN-I**.

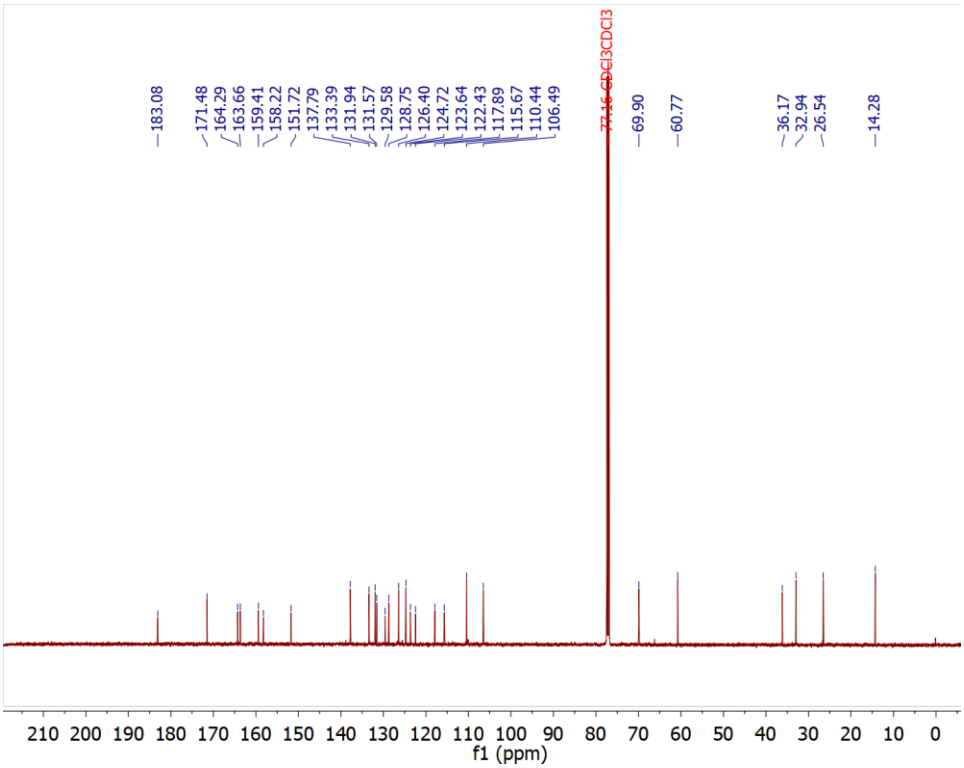


Figure S14. The ^{13}C NMR spectrum of **HN-I**.

7. Authors Contribution

Yueci Wu: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - Original Draft, Writing – Review & Editing.

Hai-Hao Han: Investigation, Writing – Original draft, Writing – Review & Editing.

Liu He: Investigation.

Yi Zang: Investigation.

Li Li: Resources.

Jia Li: Conceptualization, Investigation, Supervision, Writing – Review & Editing.

Xiao-Peng He: Conceptualization, Investigation, Supervision, Writing – Review & Editing.

Yaping Ding: Funding acquisition.

Weiguo Cao: Writing - Original Draft, Writing – Review & Editing, Supervision, Project administration, Funding acquisition.

Tony D. James: Conceptualization, Methodology, Investigation, Writing - Original Draft, Writing – Review & Editing, Supervision, Project administration, Funding acquisition.

8. References

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