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Supporting Information for

A therapeutic probe for detecting and inhibiting $ONOO^-$ in senescence

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Materials and instruments

All reagents and materials were purchased from commercial companies and used without further purification unless otherwise stated. Twice Distilled water was used in all experiments. 4-hydroxybenzaldehyde, 2'-hydroxyacetophenone, methanol (MeOH), sodium hydroxide (NaOH), H_2O_2, hydrochloric acid (HCl), petroleum ether (PE), ethyl acetate (EA), dichloromethane (DCM), triethylamine (TPA), Dimethylaminothioformyl chloride, Phosphate Buffered Saline (PBS), Ca^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Hg^{2+}, K^+, Na^+, F^-, Br^-, NO_2^-, OAc^-, PO_4^{3-}, SCN^-, HSO_3^-, $S_2O_3^{2-}$, SO_3^{2-}, SO_4^{2-}, H_2O_2, ClO^-, Ser (Serine), Asp (Asparagicacid), His (Histidine), GSH (Glutathione), Cys (Cysteine), a-D-Glu (a-D-Glucose), trypsin, cellulase, pepsase, lysozyme and $ONOO^-$. All aqueous solutions were prepared with ultra-pure water obtained from a Milli-Q water purification system (18.2 MΩ cm).

^1H NMR and ^{13}C NMR spectra were obtained on a Bruker Avance III HD 500 MHz NMR spectrometer (Germany). High resolution mass spectrometric (HRMS) analyses were measured on Aglient 6550 Q-TOF. The absorbance was recorded by ultraviolet-visible absorption spectrometry (UV-2700, Shimadzu) or microplate reader (TransGen Biotechnology). TLC analysis were carried out on silica gel plates and column chromatography was conducted over silica gel (mesh 200-300), both were purchased from the Qingdao Ocean Chemicals. Cells were photographed under the microscope (RVL-100-G, USA Discover-Echo, 60×).

The preparation process of $ONOO^-$

8.8 mL 30% H_2O_2 was dissolved in 50 mL ultra-pure water, 16 mL 5 N NaOH and 5 mL 0.04 M diethylene triamine pentaacetic acid (dissolved in 0.05 N NaOH) were added at 4 ℃, and then diluted to 100 mL ultra-pure water. The concentration of H_2O_2 in the final solution is 0.2 - 2 M, and the pH range is 12.5 - 13.0. Then add 10.8 mL of isoamyl nitrite in equal measure and stir at room temperature for 1 - 15 h. Remove isoamyl nitrite and isoamyl alcohol by cleaning the aqueous phase with chloroform or DCM. The aqueous phase removes the unreacted H_2O_2 through the active MnO_2 column.

The detection process of $ONOO^-$: Take 1.5 mL solution every 30 min and dilute it with 0.1 N NaOH to test the absorption value A at 302 nm by 500 - 1000 times UV.

$$C_{ONOO^-} = Abs_{302nm} / 1.67 \text{ (mM)}$$

Synthesis of compound FLAH

4-hydroxybenzaldehyde (20.49 mmol, 2500 mg, 1 eq), 2'-hydroxyacetophenone (22.54 mmol, 3066.57 mg, 1.1 eq) were dissolved in 50 mL methanol, and sodium hydroxide (10 eq) was slowly added to the solution. The mixture was refluxed for about 12 h. After the reaction, the mixture is cooled to room temperature. Sodium hydroxide solution (0.5 mol/L, 10 eq) and H_2O_2 solution (5 eq) were added to the mixture and stirred at room temperature for 5 h. When the reaction finished, the mixture was poured into ice water and the pH value was adjusted to neutral with hydrochloric acid, and precipitation occurred. The mixture was filtered by vacuum pressure and purified by column chromatography (PE: EA = 200: 1), yielding a light-yellow solid compound 1 (240 mg, 4.61% yield).

Preparation of Solutions of probe FLASN and Analytes

Unless otherwise stated, all tests are carried out according to the following procedures. A probe solution of **FLASN** (1.0 mM) was prepared in DMSO. After the final volume was adjusted to 10 mL with PBS buffer (90% DMSO), placed at 37℃ for 20 minutes. Then 3 mL portion was transferred to a 1 cm quartz cell to measure absorbance and fluorescence. All fluorescence measurements were made on Hitachi F4600 Fluorescence Spectrophotometer. By adding the minimum volume of sodium NaOH (0.1 M) or HCl (0.2 M), the pH of the solution changed slightly.

Determination of the detection limit

The detection limit was determined using the fluorescence titration. To get the slope, the fluorescence intensity (at 525 nm) was plotted as the $ONOO^-$ concentration increased. The detection limit was obtained using the equation:

$$\text{Detection Limit} = 3\sigma/k$$

Where σ is the standard deviation of blank measurement (σ = 5.84187), k is the slope between the fluorescence intensity versus the concentrations of $ONOO^-$ (k = 3886.9), so the detection limit is 4.5 nM.

Culture and preparation of HeLa, HL 7702 and Cos 7 cells

HeLa, HL 7702 and Cos 7 cells were cultured in DMEM (Dulbecco's modified Eagle's medium) with 10% fetal bovine serum (FBS) in 5% CO_2 and 95% air at 37 ℃. Before the experiment, HeLa, HL 7702 and Cos 7 cells were inoculated in a 35 mm glass-bottomed culture dish with a density of 2×10^5 cells per dish, and then cultured in

2 mL culture medium at 37℃ in an incubator containing 5% CO_2 and 95% air for 24 hours. During this period, cells will adhere to the glass surface. In use, HeLa, HL 7702 and Cos 7 cells were treated with **FLASN** at 37℃ for 5 minutes.

Cytotoxicity assays

Cells were inoculated into 96-well plates, and probe **FLASN** (95% DMEM and 5% DMSO) of 0, 1, 2, 5, 10, 30, and 40 μM (final concentration) were added respectively. Subsequently, the cells were cultured at 37 °C in an atmosphere of CO_2 (5%) and air (95%) for 24 hours. Then cells were washed with PBS buffer and DMEM medium was added. Next, MTT (10 μL, 5 mg/mL) was injected into each well and incubated for 4 hours. Treatment with sodium dodecyl sulfate solution (100 μL) in H_2O-DMF mixture produced purple methyl. The viability of cells was determined by assuming that the viability of cells without **FLASN** was 100%.

Preparation of cell imaging experiment.

The HeLa cells were plated at 1×10^5 cells / mL suspension in μ-slide 8 well and allowed to culture overnight, respectively. Then the cells were treated with $ONOO^-$ (5 and 10 μM) for 30 min with fresh culture medium. HeLa cells untreated with $ONOO^-$ were used as control. 10 μM of **FLASN** was added into the cell groups for 5 min. The cell images were obtained using the microscope (RVL-100-G, USA Discover-Echo, 60×).

The HL 7702 cells were plated at 1×10^5 cells / mL suspension in μ-slide 8 well and allowed to culture overnight, respectively. Then the cells were treated with metformin

(20 and 40 mM) for 4 hours with fresh culture medium. HL 7702 cells untreated with metformin were used as control. 10 μM of **FLASN** was added into the cell groups for 5 min. The cell images were obtained using the microscope (RVL-100-G, USA Discover-Echo, 60×).

The Cos 7 cells were plated at 1×10^5 cells / mL suspension in μ-slide 8 well and allowed to culture overnight, respectively. Senescent Cos 7 cells were obtained by replicative passage, and normal Cos 7 cells were used as control. 10 μM of **FLASN** was added into the cell groups for 5 min. The cell images were obtained using the microscope (RVL-100-G, USA Discover-Echo, 60×).

X-gal staining of cells

A senescence-associated β-galactosidase staining kit (C0602, Beyotime, Jiangsu, China) was used to detect cellular senescence. Cos 7 were rinsed once with PBS or Hanks balanced salt solution (PBS) and fixed with 1 mL of β-galactosidase stationary liquid at room temperature for 10 min. Then, the cell suspension underwent centrifugation, after which the stationary liquid was removed, and the samples were rinsed with PBS three times (3 min each time). Remove PBS, add 1 ml of dye solution to each well, 37 º C incubated overnight. Then the stained cells were photographed under the microscope (RVL-100-G, USA Discover-Echo, 40×), and the aging cells turned blue.

Preparation of zebrafish imaging experiment.

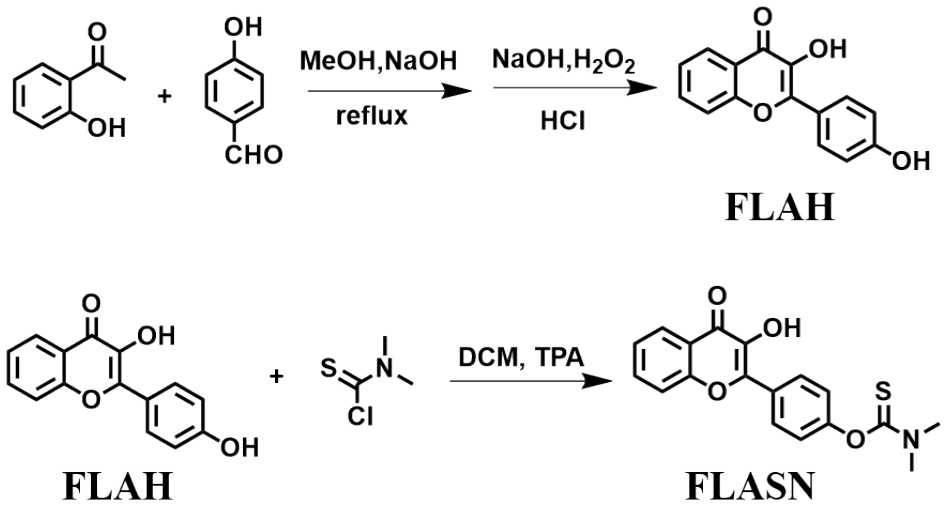
The zebrafish were allowed to culture overnight, respectively. Then the zebrafish were treated with $ONOO^-$ (15, 20, 30, 40 μM) for 30 min with fresh culture medium.

zebrafish untreated with $ONOO^-$ were used as control. 10 μM of **FLASN** was added into the zebrafish groups for 5 min. The zebrafish images were obtained using the microscope (RVL-100-G, USA Discover-Echo, 2×).

The zebrafish were allowed to culture overnight, respectively. Then zebrafish were treated with metformin (20 and 40 mM) for 4 hours with fresh culture medium. zebrafish untreated with metformin were used as control. Metformin is a mild mitochondrial complex I inhibitor, and could activate AMPK by increasing the generation of $ONOO^-$. On the other hand, the major target of metformin is liver, and accordingly zebrafish were used for the investigation of endogenous $ONOO^-$ *in vivo*.[16] 10 μM of **FLASN** was added into the cell groups for 5 min. The cell images were obtained using the microscope (RVL-100-G, USA Discover-Echo, 2×).

Statistical Analysis

The data were expressed as mean ± SD. The error bar represents the standard deviation, n = 3. Statistical calculation of experimental data was using the One-way ANOVA statistical analysis. The data were classified with p values and denoted by (*) for $p < 0.1$, (**) for $p < 0.01$, (***) for $p < 0.001$, (ns) for $p > 0.05$ no significance.



Scheme S1. Synthetic route of probe FLASN.

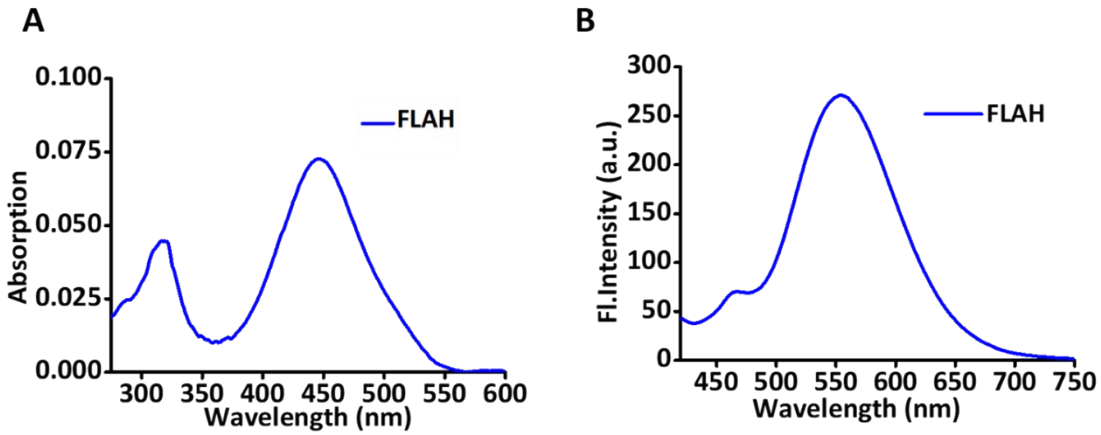


Fig. S1. (A) Absorption spectra of FLASN (5 µM), FLAH (5 µM) in the absence and presence of $ONOO^-$ (0.06 µM). (B) Fluorescence spectra of FLASN (5 µM), FLAH (5 µM) in the absence and presence of $ONOO^-$ (0.06 µM).

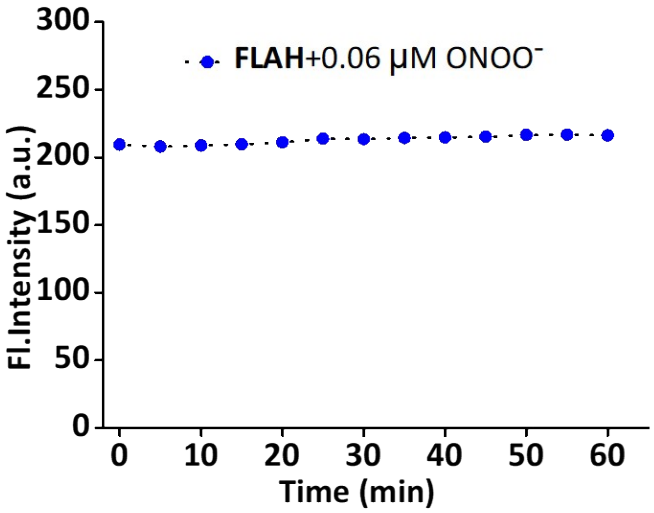


Fig. S2. Time-dependent fluorescence intensity changes of FLAH (5 μM) in the presence of $ONOO^-$ (0.06 μM).

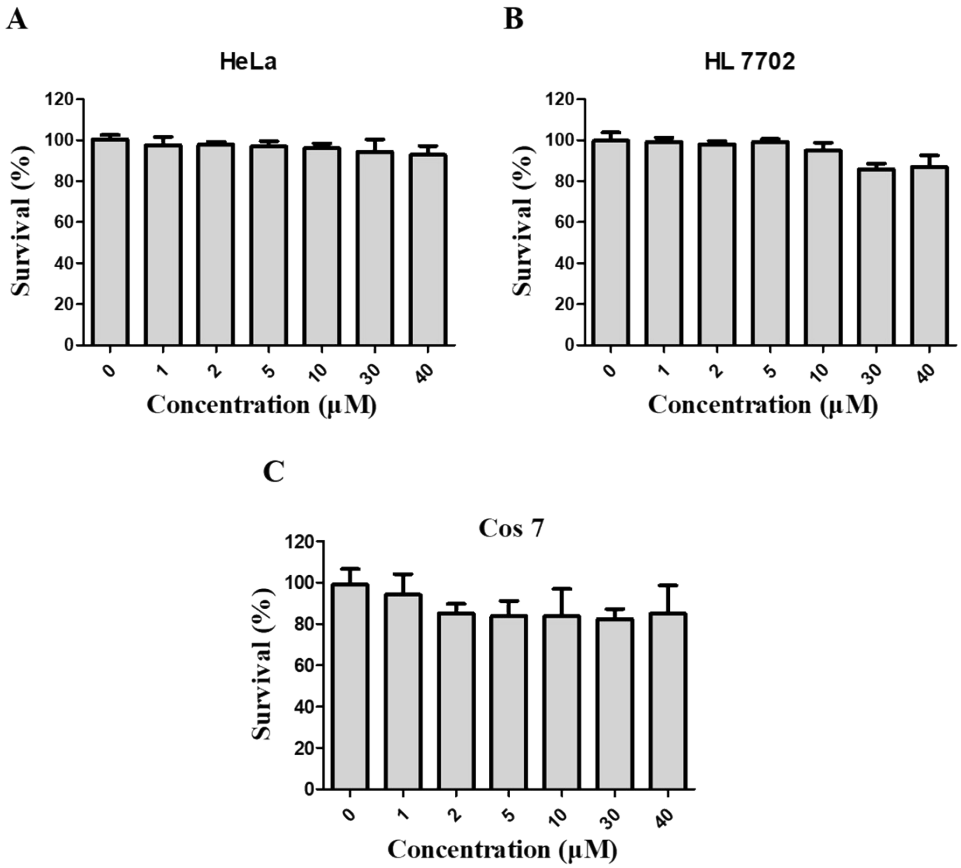


Fig. S3. Cytotoxicity of probe FLASN in HeLa, HL 7702 and Cos 7 cells.

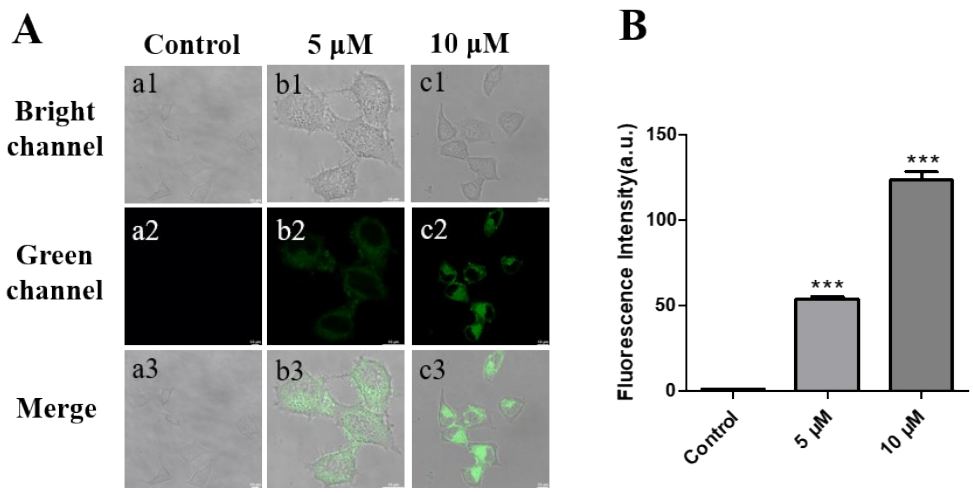


Fig. S4. (A) FLASN was applied to image exogenous $ONOO^-$ in HeLa cells. (a1 - a3) Control group was stained with 10 μM probe, cells were incubated with $ONOO^-$ at different concentrations for 30 min, (b1 - b3) 5 μM, (c1 - c3) 10 μM, and then probe FLASN (10 μM) was added. (B) Fluorescence intensity diagram of (A).

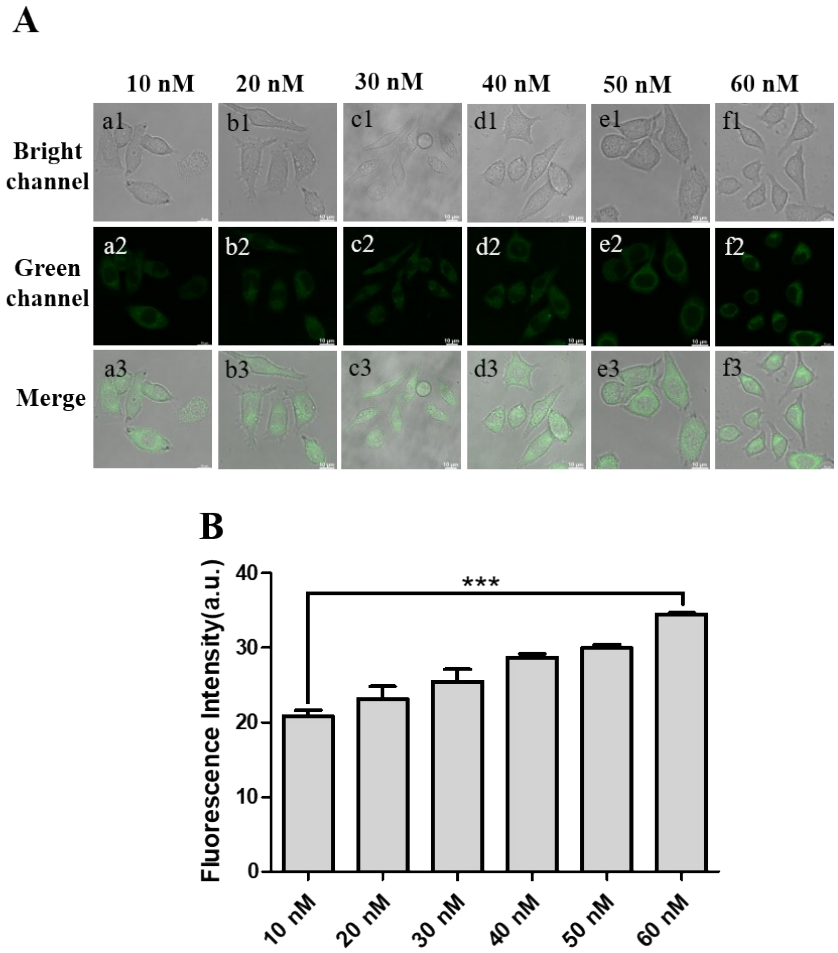


Fig. S5. (A) Exogenous fluorescence imaging of FLASN in HeLa cells. (a – f) HeLa cells incubated with $ONOO^-$ (10, 20, 30, 40, 50, and 60 nM) for 30 min, and then probe FLASN (5 μM) was added. (B) Fluorescence intensity diagram from (A). Scale bar = 10 μm. The data were classified with p values and denoted by ***p < 0.001.

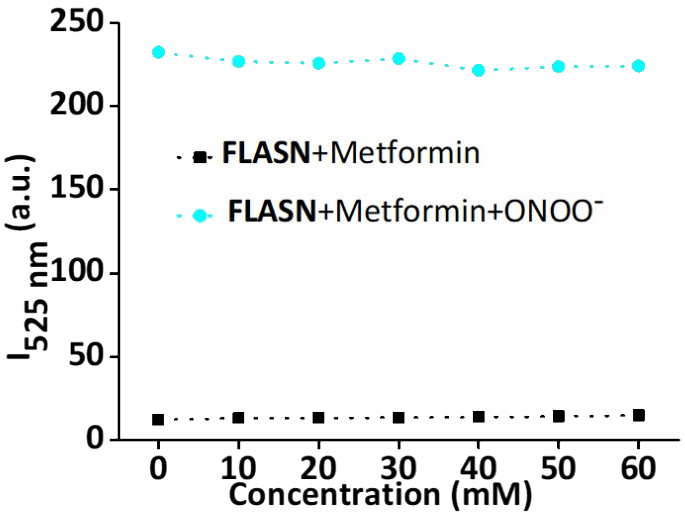


Fig. S6. Fluorescence spectra of FLASN (5 μM) on different concentrations of metformin (0 – 60 mM) without or with $ONOO^-$ at 525 nm.

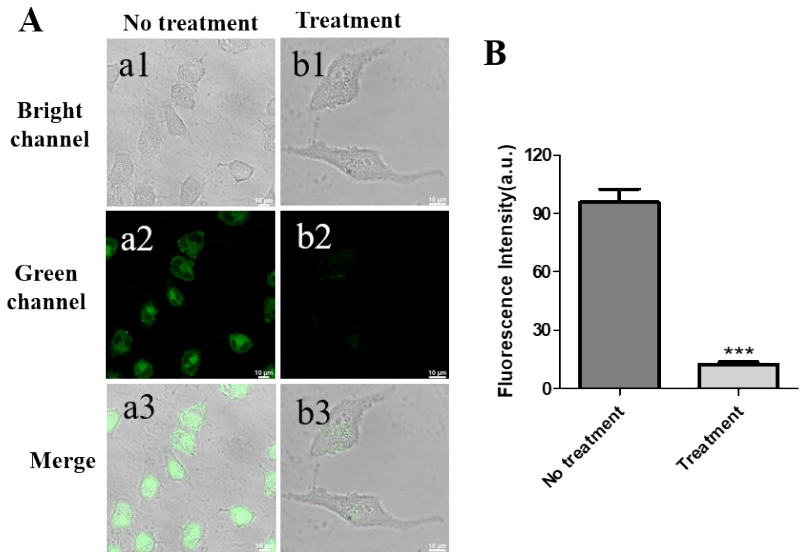


Fig. S7. (A) Fluorescence imaging of treated and untreated HL 7702 cells. (a1 - a3) Cells were not treated with FLASN. (b1 - b3) Cells were treated with FLASN for two days, and then probe FLASN was added. (B) Fluorescence intensity diagram from (A).

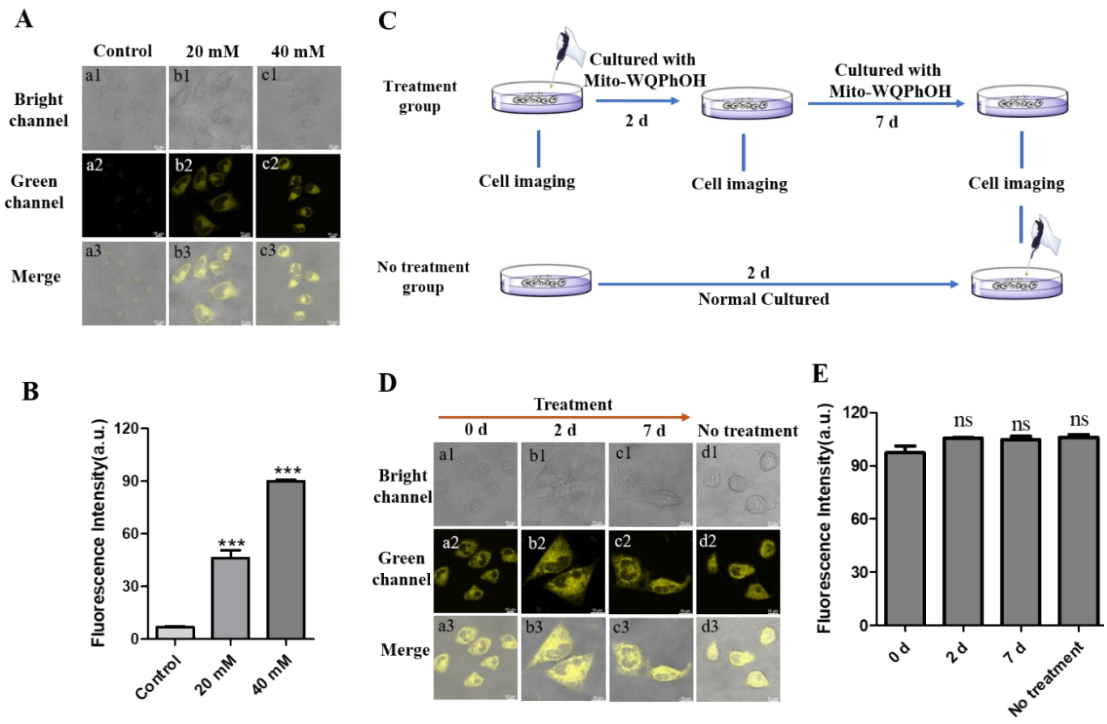


Fig. S8. Fluorescence imaging of Mito-WQPhOH. (A) Fluorescence imaging of **Mito-WQPhOH** in HL 7702 cells. (a1 - a3) Control group was stained with 10 μM **Mito-WQPhOH**, cells were incubated with different concentrations of metformin for 4 h, (b1 - b3) 20 mM, (c1 - c3) 40 mM, and then **Mito-WQPhOH** (10 μM) was added. (B) Fluorescence intensity diagram from (A). (C) Graphical explanation of the treatment of endogenous $ONOO^-$ in HL 7702 cells with **Mito-WQPhOH**. (D) HL 7702 cells were incubated with metformin (40 mM) for 4 h, and then **Mito-WQPhOH** (10 μM) was added for time tracking of treatment. (a1 - a3) Cells were treated with **Mito-WQPhOH** for 0 day, (b1 - b3) Cells were treated with **Mito-WQPhOH** for 2 days, (c1 - c3) Cells were treated with **Mito-WQPhOH** for 7 days, (d1 - d3) Cells were not treated with **Mito-WQPhOH**. (E) Fluorescence intensity diagram from (D). Scale bar = 10 μm. The data were classified with p values and denoted by ***$p < 0.001$, ns $p > 0.05$ no significance.

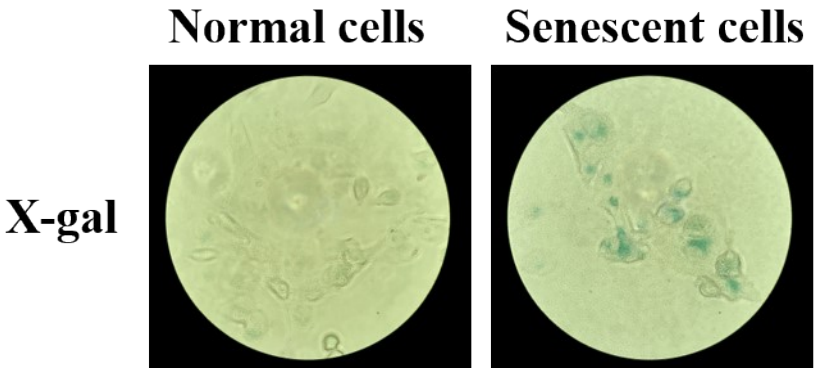


Fig. S9. X-gal staining of Cos 7 cells.

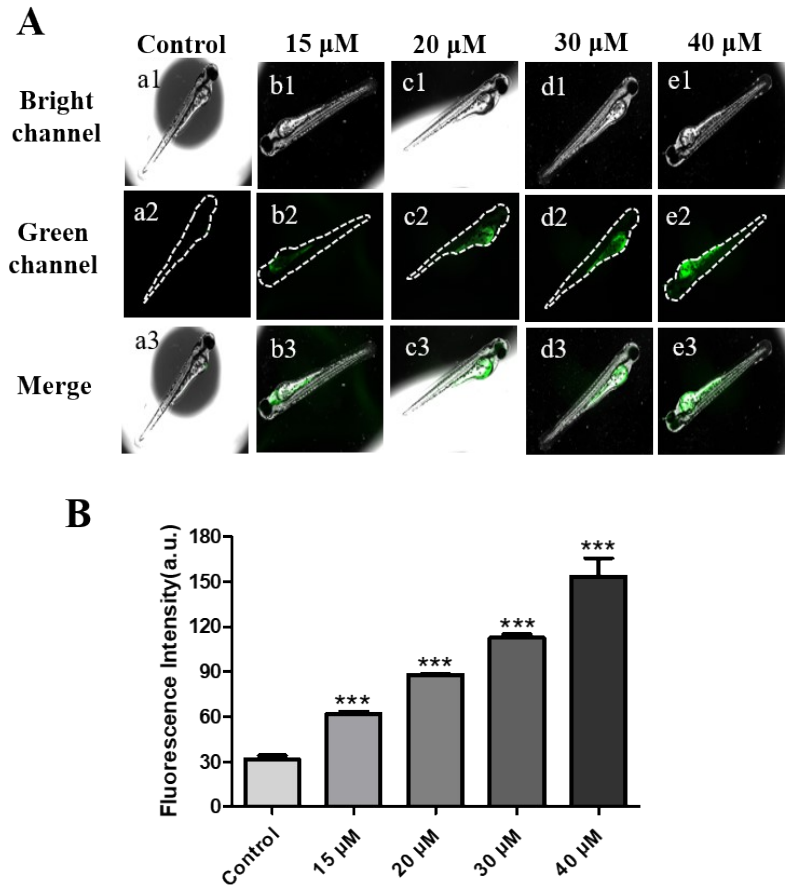


Fig. S10. (A) Exogenous fluorescence imaging of FLASN in zebrafish. (a - e) Zebrafish were stained with 10 μM probe and incubated with $ONOO^-$ (0, 15, 20, 30, and 40 μM) for 30 min, and then probe FLASN (10 μM) was added. (B) Fluorescence intensity diagram from (A).

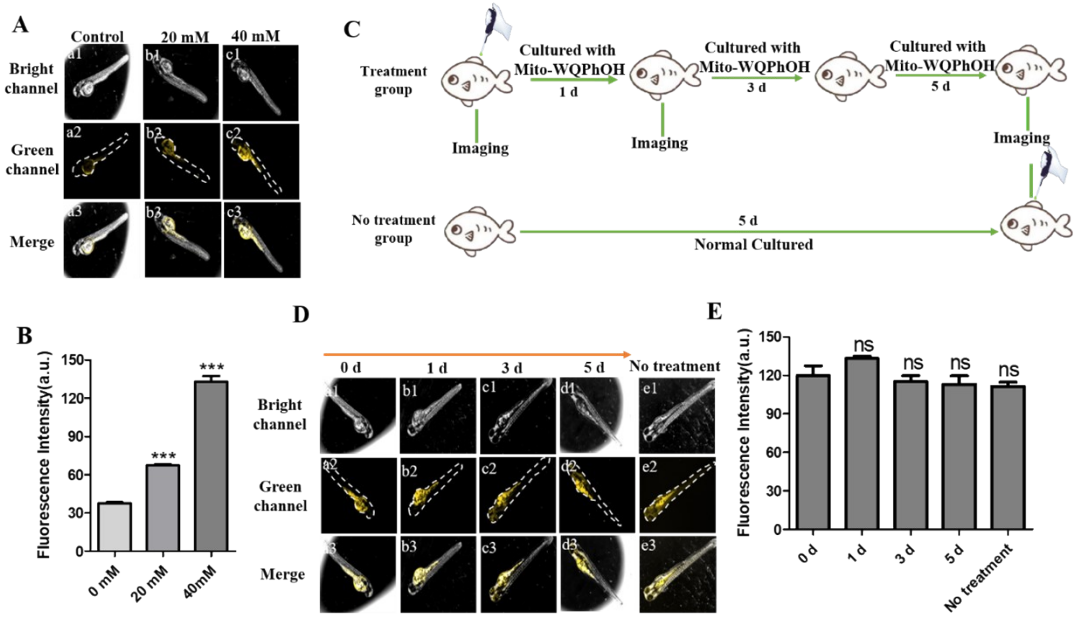


Fig. S11. **Endogenous fluorescence imaging of Mito-WQPhOH**. (A) Endogenous fluorescence imaging of **Mito-WQPhOH** in zebrafish. (a - c) Zebrafish were incubated with metformin (0, 20, and 40 mM) for 4 h, and then probe **Mito-WQPhOH** (10 μM) was added. (B) Fluorescence intensity diagram from (A). (C) Graphical explanation of the treatment of zebrafish with probe **Mito-WQPhOH**. (D) Zebrafish were incubated with metformin (40 mM) for 4 h, and then **Mito-WQPhOH** (10 μM) was added for time tracking of treatment. (a1 - a3) Zebrafish were treated with **Mito-WQPhOH** for 0 day, (b1 - b3) Zebrafish were treated with **Mito-WQPhOH** for 1 day，(c1 - c3) Zebrafish were treated with **Mito-WQPhOH** for 3 days, (d1 - d3) Zebrafish were treated with **Mito-WQPhOH** for 5 days，(e1 - e3) Zebrafish were not treated with **Mito-WQPhOH**. (E) Fluorescence intensity diagram from (D). Scale bar = 10 μm.The data were classified with p values and denoted by ***$p < 0.001$, ns $p > 0.05$ no significance.

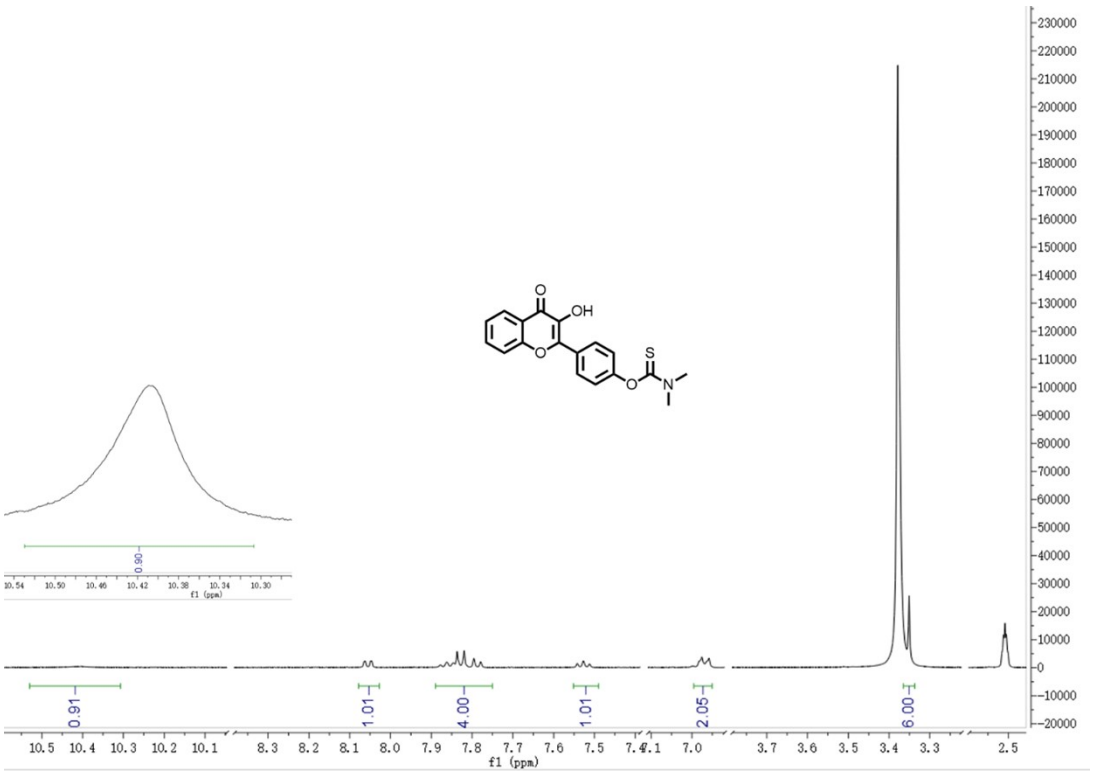


Fig. S12. ^{1}H NMR (DMSO-d6) spectrum of probe FLASN.

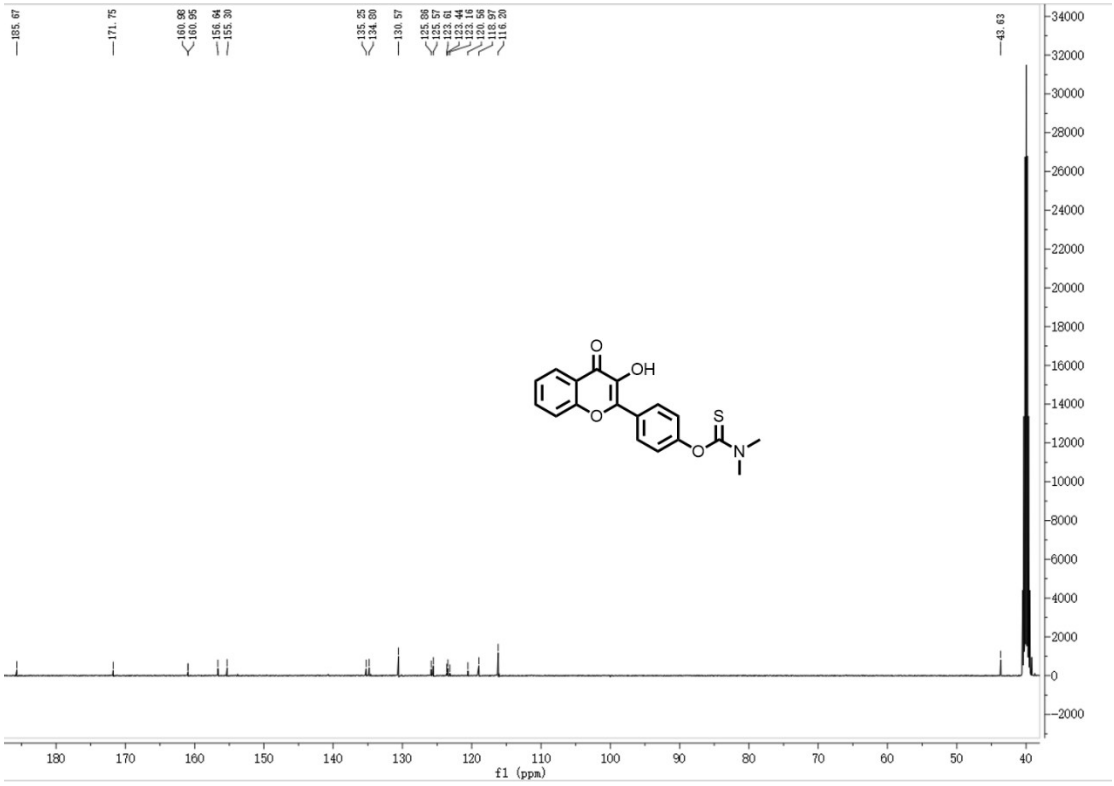


Fig. S13. ^{13}C NMR (DMSO-d6) spectrum of probe FLASN.

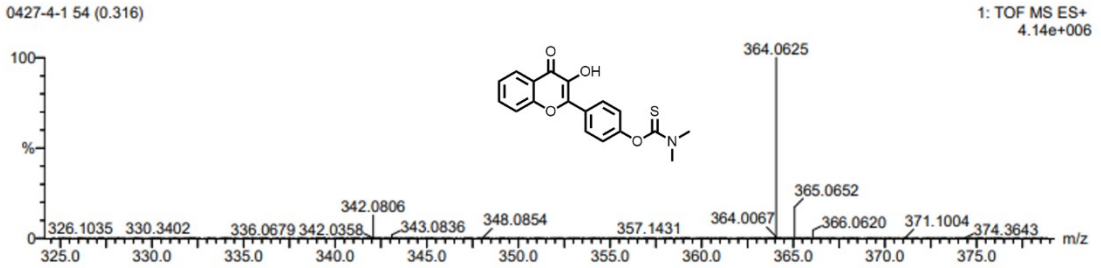


Fig. S14. HRMS (ESI) spectrum of probe FLASN, $[M + Na]^+$, 364.0625.

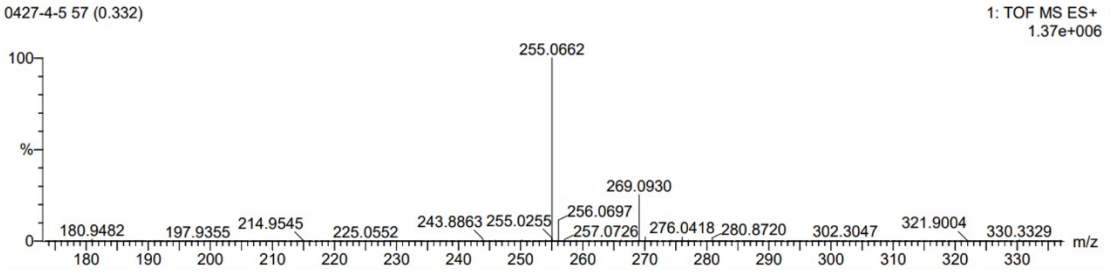


Fig. S15. HRMS (ESI) spectrum of the reaction products of probe FLASN with $ONOO^-$, M^+, 255.0662.

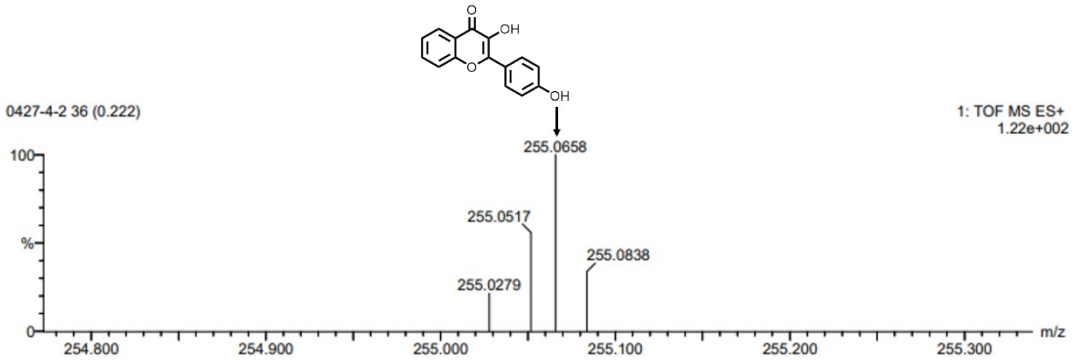


Fig. S16. HRMS (ESI) spectrum of FLAH, M^+, 255.0658.