Supporting Information

Fluorescent Probe for the Imaging of Superoxide and Peroxynitrite during Drug-induced Liver Injury

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Table of contents

1. Materials and instruments .............................................................. S2
2. Preparation of ROS/RNS ............................................................... S2
3. UV-Vis and fluorescence analysis .................................................. S3
4. Mass spectroscopic analysis ......................................................... S8
5. Protocols for cell culture ............................................................... S23
6. Fluorescence imaging in live cells and MTT assay .............................. S23
7. Near-infrared fluorescence imaging of APAP-induced injury in mice .... S30
8. Two-photon fluorescence imaging of APAP-induced injury in mice .... S30
9. Hematoxylin and eosin (H&E) staining of main organs in mice ........ S30
10. Synthesis of probe LW-OTf ......................................................... S32
11. NMR spectra .............................................................................. S34
12. Characterisation of LW-OTf triflate salt ........................................ S36
13. Fluorescence “turn on” mechanisms and selectivity of LW-OTf and other hemicyanine-xanthene dyes ....................................................... S40
14. Statistical analysis ...................................................................... S40
15. References .................................................................................. S41
1. Materials and instruments

2-Methoxyestradiol (2-ME) was purchased from Aladdin Industrial Corporation. 3-Morpholinosydnonimine hydrochloride (SIN-1) was purchased from Sigma-Aldrich Co. LLC. 4-Acetamidophenol (APAP) and butylated hydroxyanisole (BHA) were purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. LC-MS analysis were performed using an Agilent QTOF 6545 with Jetstream ESI spray source coupled to an Agilent 1260 Infinity II Quat pump HPLC with 1260 autosampler, column oven compartment and variable wavelength detector (VWD). Fluorescence spectra were obtained with a FLS-1000-SS-STM spectrometer (Edinburgh Instruments Ltd., England). Absorption spectra were recorded on a BMG Labtech CLARIOstar®. MTT assay was performed using a Triturus microplate reader. Confocal imaging was performed on Leica SP8 high-resolution fluorescence microscope. The two-photon fluorescence images were taken using a LSM 880 confocal laser scanning microscope (Zeiss Co., Ltd. Germany). Two-photon fluorescence response in Figure S2 was performed with Zeiss 880 NLO microscope and the supporting software ZEN 2 lite using “Lambda Mode” in imaging setup. Fluorescence imaging in mice was performed on a Caliper IVIS Lumina Series III. Hematoxylin-eosin (H&E) staining images were obtained with optical microscope (NIKON Eclipse ci). Nuclear Magnetic Resonance (NMR) spectroscopy experiments were performed in deuterated solvent at 298 K on either a Bruker Avance 500 MHz spectrometer or an Agilent ProPulse 500 MHz spectrometer.

2. Preparation of ROS/RNS

\( \text{O}_2^{•−} \)
O₂⁻ was produced from KO₂ in dry DMSO by an ultrasonic method. The concentration of O₂⁻ was determined from the absorption at 250 nm \((ε = 2682 \text{ M}^{-1} \text{ cm}^{-1})\).

\( \text{ONOO}^− \)
0.6 M NaNO₂, 0.6 M HCl and 0.7 M H₂O₂ were added simultaneously to a 3 M NaOH solution at 0 °C. The concentration of peroxynitrite was determined using extinction coefficient of 1670 M⁻¹ cm⁻¹ at 302 nm in 0.1 M NaOH (aq.).

\( \text{H}_2\text{O}_2 \)
H₂O₂ solutions were accessed by dilution of 30% hydrogen peroxide aqueous solution, the concentration was determined from the absorption at 240 nm \((ε = 43.6 \text{ M}^{-1} \text{ cm}^{-1})\).

\( \text{TBHP} \)
TBHP solutions were accessed by dilution of 70 % tert-butyl hydroperoxide aqueous solution.

\( \text{•OH} \)
•OH (hydroxyl radical) was generated by the Fenton reaction of FeCl₂ (1.0 mM) and H₂O₂ (200 μM) in deionised water.

\( \text{NO} \)
NO (Nitric oxide) was obtained from a stock solution prepared by sodium nitroprusside.

\( \text{¹O}_2 \)
¹O₂ (Singlet oxygen) was prepared by the NaClO-H₂O₂ system.
3. UV-Vis and fluorescence analysis

3.1 Experimental procedure for fluorescence measurement of O$_2^•$− (Figure 1).
Various concentrations of O$_2^•$− (0–25 μM) were added to probe LW-OTf in DMSO. After 1 min the mixture was diluted to 1 mL with PBS buffer (10 mM, pH 7.4) and incubated for 10 min before measurements. The final concentration of LW-OTf in PBS buffer solution (with 1% DMSO) was 2.4 μM. $\lambda_{ex/em} = 675/710$ nm.

3.2 Experimental procedure for fluorescence measurement of ONOO$^-$ (Figure 2).
O$_2^•$− (5 μM) was added to probe LW-OTf in DMSO. After 1 min, various concentrations of ONOO$^-$ (0–36 μM) were added to the solution, and after an additional 1 min the mixture was diluted to 1 mL with PBS buffer (10 mM, pH 7.4) and incubated for 7 min. The final concentration of LW-OTf in PBS buffer solution (with 1% DMSO) was 2.4 μM. $\lambda_{ex/em} = 360/461$ nm.

3.3 Experimental procedures for absorption spectra (Figure S1).

**Probe:** Probe LW-OTf (10 μM) was diluted to 1 mL with PBS buffer (10 mM, pH 7.4, 1% DMSO).

**Probe + 20 μM O$_2^•$−:** O$_2^•$− (20 μM) was added to LW-OTf in DMSO, and after 1 min the mixture was diluted to 1 mL with PBS buffer (10 mM, pH 7.4) and incubated for 10 min. The final concentration of LW-OTf in PBS buffer solution (with 1% DMSO) was 10 μM.

**Probe + 20 μM O$_2^•$− + 17.5 μM ONOO$^-$:** O$_2^•$− (20 μM) was added to LW-OTf in DMSO, and after 1 min ONOO$^-$ (17.5 μM) was added. After an additional 1 min the mixture was diluted to 1 mL with PBS buffer (10 mM, pH 7.4) and incubated for 7 min. The final concentration of LW-OTf in PBS buffer solution (with 1% DMSO) was 10 μM.

![Figure S1. Absorption spectra of probe LW-OTf (10 μM) with and without O$_2^•$− (20 μM), and LW-OTf (10 μM) with the addition of O$_2^•$− (20 μM) then addition of ONOO$^-$ (17.5 μM).](image-url)
3.4 Experimental procedures for two-photon experiments (Figure S2).

**Probe + 5 μM O$_2^-$**: O$_2^-$ (5 μM) was added to probe LW-OTf in DMSO, and after 1 min the mixture was diluted to 1 mL with PBS buffer (10 mM, pH 7.4) and incubated for 10 min. The final concentration of LW-OTf in PBS buffer solution (with 1% DMSO) was 2.4 μM.

**Probe + 5 μM O$_2^-$ + 4.2 μM ONOO$^-$**: O$_2^-$ (5 μM) was added to probe LW-OTf in DMSO, and after 1 min ONOO$^-$ (4.2 μM) was added. After an additional 1 min the mixture was diluted to 1 mL with PBS buffer (10 mM, pH 7.4) and incubated for 7 min. The final concentration of LW-OTf in PBS buffer solution (with 1% DMSO) was 2.4 μM.

![Figure S2](image-url)

**Figure S2.** Two-photon fluorescence response of probe LW-OTf (2.4 μM) with O$_2^-$ (5 μM), and LW-OTf (2.4 μM) with the addition of O$_2^-$ (5 μM) then addition of ONOO$^-$ (4.2 μM). $\lambda_{ex} = 720$ nm, collected at 400-568 nm.

3.5 Experimental procedures for selectivity screen towards O$_2^-$ (Figure 3a)

**Blank:** Probe LW-OTf (2.4 μM) was diluted to 1 mL with PBS buffer (10 mM, pH 7.4).

O$_2^-$: O$_2^-$ (18 μM) was added to LW-OTf in DMSO, and after 1 min the mixture was diluted to 1 mL with PBS buffer (10 mM, pH 7.4) and incubated for 10 min. The final concentration of LW-OTf in PBS buffer solution (with 1% DMSO) was 2.4 μM.

Other ROS/RNS or metal ions: The appropriate ROS/RNS or metal ion was added to LW-OTf in DMSO, and after 1 min the mixture was diluted to 1 mL with PBS buffer (10 mM, pH 7.4) and incubated for 10 min. The final concentration of LW-OTf in PBS buffer solution (with 1% DMSO) was 2.4 μM.

3.6 Experimental of pH titrations of LW-OTf with O$_2^-$ (Figure S3)

**Blank:** Probe LW-OTf (2.4 μM) was diluted to 1 mL with PBS buffer (10 mM) at various pH’s and the mixtures were incubated for 10 min.

O$_2^-$: O$_2^-$ (18 μM) was added to LW-OTf in DMSO, and after 1 min the mixture was diluted to 1 mL with PBS buffer (10 mM) at various pH’s and incubated for 10 min. The final concentration of LW-OTf in PBS buffer solution (with 1% DMSO) was 2.4 μM.
3.7 Time-course fluorescence measurements of LW-OTf and O$_2$\textsuperscript{−} (Figure S4).

**Blank:** Probe LW-OTf (2.4 μM) was diluted to 1 mL with PBS buffer (10 mM, pH 7.4, 1% DMSO). 200 μL were transferred into a microcuvette and fluorescence measurements were performed immediately.

O$_2$\textsuperscript{−}: O$_2$\textsuperscript{−} (18 μM) was added to LW-OTf in DMSO, and after 1 min, the mixture was diluted to 1 mL with PBS buffer (10 mM, pH 7.4). 200 μL were transferred into a microcuvette and fluorescence measurements were performed immediately. The final concentration of LW-OTf in PBS buffer solution (with 1% DMSO) was 2.4 μM.

![Figure S4](image-url)

**Figure S4.** Time course of changes in the fluorescence of probe LW-OTf (2.4 μM, black curve), and probe LW-OTf (2.4 μM) followed by the addition of O$_2$\textsuperscript{−} (18 μM, red curve). $\lambda_{\text{ex/em}} = 675/710$ nm.

3.8 Experimental procedures for the selectivity screen towards ONOO$^-$ (Figure 3b)
**Blank:** $O_2^-$ (5 μM) was added to LW-OTf in DMSO, and after 1 min the mixture was diluted to 1 mL with PBS buffer (10 mM, pH 7.4) and incubated for 7 min. The final concentration of LW-OTf in PBS buffer solution (with 1% DMSO) was 2.4 μM.

**ONOO**: $O_2^-$ (5 μM) was added to LW-OTf in DMSO, and after 1 min ONOO$^-$ (4.2 μM) was added to the solution. After an additional 1 min, the mixture was diluted to 1 mL with PBS buffer (10 mM, pH 7.4) and incubated for 7 min. The final concentration of LW-OTf in PBS buffer solution (with 1% DMSO) was 2.4 μM.

**Other ROS/RNS or metal ions:** $O_2^-$ (5 μM) was added to LW-OTf in DMSO, and after 1 min. the appropriate ROS/RNS or metal ion was added. After an additional 1 min, the mixture was diluted to 1 mL with PBS buffer (10 mM, pH 7.4) and incubated for 7 min. The final concentration of LW-OTf in PBS buffer solution (with 1% DMSO) was 2.4 μM.

**3.9 Experimental procedures for pH titrations of LW-OTf with ONOO**

**Blank:** $O_2^-$ (5 μM) was added to LW-OTf in DMSO, and after 1 min the mixture was diluted to 1 mL with PBS buffer a various pH’s (10 mM) and incubated for 7 min. The final concentration of LW-OTf in PBS buffer solution (with 1% DMSO) was 2.4 μM.

**ONOO**: $O_2^-$ (5 μM) was added to LW-OTf in DMSO, and after 1 min ONOO$^-$ (4.2 μM) was added. After an additional 1 min, the mixture was diluted to 1 mL with PBS buffer (10 mM, pH 7.4). The final concentration of LW-OTf in PBS buffer solution (with 1% DMSO) was 2.4 μM.

![Fluorescence spectra of probe LW-OTf (2.4 μM) with $O_2^-$ (5 μM) (black squares), and LW-OTf (2.4 μM) with $O_2^-$ (5 μM) followed by the addition of ONOO$^-$ (4.2 μM) (blue circles) at various pH’s. $\lambda_{ex/em}$ = 360/461 nm.](image)

**3.10 Time-course fluorescence measurements of LW-OTf with ONOO**

**Blank:** $O_2^-$ (5 μM) was added to LW-OTf in DMSO, and after 1 min the mixture was diluted to 1 mL with PBS buffer (10 mM, pH 7.4). The final concentration of LW-OTf in PBS buffer solution (with 1% DMSO) was 2.4 μM. 200 μL were transferred into a microcuvette, and fluorescence measurements were performed immediately.

**ONOO**: $O_2^-$ (5 μM) was added to LW-OTf in DMSO, and after 1 min ONOO$^-$ (4.2 μM) was added. After an additional 1 min, the mixture was diluted to 1 mL with PBS buffer (10 mM, pH 7.4). The final concentration of LW-OTf in PBS buffer solution (with 1% DMSO) was 2.4 μM.
concentration of **LW-OTf** in PBS buffer solution (with 1% DMSO) was 2.4 μM. 200 μL were transferred into a microcuvette, and fluorescence measurements were performed immediately.

**Figure S6.** Time course of changes for the fluorescence of probe **LW-OTf** (2.4 μM) with **O₂•−** (5 μM), and **LW-OTf** (2.4 μM) with **O₂•−** (5 μM) followed by the addition of **ONOO−** (4.2 μM). \( \lambda_{\text{ex/em}} = 360/461 \) nm.
4. Mass spectroscopic analysis

Scheme S1. Proposed mechanism for the reaction of probe LW-OTf upon sequential additions of O$_2^-$ then ONOO$^-$.

Probe LW-OTf (4.8 mM, in DMSO) was diluted to 19 μM in methanol (Figure S7), then O$_2^-$ (60 μM) was added (Figure S8), followed by addition of ONOO$^-$ (20 μM) (Figure S9 and S10).
Walkup MS Report

Data File: xtl-01-002_Pos_LoopInection_MS_09650.d
Sample Name: xtl-01-002
Sample Type: Sample
Instrument Name: 6545 QToF
Acq Method: Pos_LoopInection_MS.m
IRM Calibration Status: Unknown
DA Method: Pos_LoopInjection_MS.m

Sample Group: Unknown
Walkup Sample: Unknown
Walkup Method: Pos_LoopInjection_MS
Description: C29H29F3N4O6
Wallup Method: Unknown
Description: Positive mode ionization using loop injection with common organic molecule isotope model
Stream Name: LC 1
Acquisition SW Version: 6.200 series TOF/EMS series Q-TOF B.06.00 (B0044.0)

Figure 1: Base peak chromatogram

User Chromatogram Peak List

| RT (min) | Area (%) | Area Sum (%) | Base Peak (m/z) | Width (min) |
|----------|----------|--------------|----------------|-------------|
| 0.78     | 132989282 | 100.00       | 544.1341       | 0.189       |

Compound Table

| Compound Label | RT (min) | Observed mass (m/z) | Neutral observed mass (Da) | Theoretical mass (Da) | Mass error (ppm) | Isotope match score (%) |
|----------------|----------|---------------------|---------------------------|-----------------------|------------------|-------------------------|
| Cpd 1: C29 H29 F3 N4 O5 | 0.73 | 544.1774 | 544.1779 | 544.1769 | 1.77 | 97.73 |

Mass errors of between -5.00 and 5.00 ppm with isotope match score above 94% are considered confirmation of molecular formula.
Figure S7. HRMS of probe LW-OTf.
Walkup MS Report

Data File: NTR + superoxide_Pos_Smina_MS_09611.d
Sample Name: NTR + superoxide
Sample Type: Sample
Position: P1-46
Instrument Name: 6545 QToF
User Name: Xue Tian
Acq Method: Pos_Smina_MS.m
Acquired Time: 06/01/2020 15:46:48
DA Method: Pos_Smina_MS.m

Sample Group: Info.
Walkup Sample: W
Description: C28H30N2O2
Wallup Method: Positive mode ionization using
Wallup Method: C18 column chromatography

Stream Name: LC 1
Acquisition SW Version: 2.00 series TOF/5000 series Q-Tof B.09.00 (804/4.0)

Figure 1: Base peak chromatogram

User Chromatogram Peak List

| RT  (min) | Area    | Area % | Area Sum (%) | Base Peak (m/z) | Width (min) |
|---------|---------|--------|--------------|----------------|-------------|
| 0.60    | 19817749| 6.51   | 3.21         | 157.0350       | 0.069       |
| 0.85    | 16377640| 6.02   | 3.06         | 474.2636       | 0.143       |
| 4.03    | 19202026| 6.15   | 3.03         | 149.0527       | 0.351       |
| 4.29    | 26055200| 15.65  | 7.73         | 185.1151       | 0.139       |
| 4.66    | 16413979| 100.00 | 49.78        | 544.1895       | 0.174       |
| 5.07    | 22898465| 12.78  | 5.97         | 743.3134       | 0.124       |
| 5.15    | 46299935| 37.87  | 19.73        | 1755.6421      | 0.189       |
| 5.45    | 12413818| 7.47   | 3.68         | 441.3003       | 0.130       |
| 5.58    | 13149589| 8.10   | 4.99         | 441.3003       | 0.394       |
| 6.28    | 18499194| 11.13  | 5.49         | 172.3779       | 0.409       |

Compound Table

| Compound Label | RT (min) | Observed mass (m/z) | Neutral observed mass (Da) | Theoretical mass (Da) | Mass error (ppm) | Isotope match score (%) |
|----------------|---------|---------------------|---------------------------|-----------------------|-----------------|------------------------|
| C28H30N2O2 D2 | 4.52    | 412.2282            | 412.2288                  | 412.2277              | 0.27            | 95.45                  |

Mass errors of between 5.00 and 1.00 ppm with isotope match scores above 60% are considered confirmation of molecular formula.

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Page 1 of 2
Figure S8. LC-MS of LW-OTf + $O_2^{−}$, showing cleavage of LW-OTf to the related phenol.
Walkup MS Report

Data File: nir + superoxide +aresol1_Pos_Sims_MS_09624.d  Sample Name: nir + superoxide +aresol1
Sample Type: Sample  Position: PI-AS
Instrument Name: 6545 QToF  User Name: Xue Tian
Acq Method: Pos_Sims_MS.m  Acquired Time: 1/9/2020 5:06:45 PM
IRM Calibration Status: 75/240  DA Method: Pos_Sims_MS.m
Comment:

Sample Group:  Walkup Sample:  Info:  Walkup Method: Pos_Sims_C18
Description:  Walkup Method:  Positive mode ionization using  Description: C18 column chromatography

Stream Name: LC 1  Acquisition SW Version: 6200 series TOF/TOF series Q-TOF B.09.00 (B644.0)

Figure 1: Base peak chromatogram

User Chromatogram Peak List

| RT (min) | Area | Area % | Area Sum (%) | Base Peak (m/z) | Width (min) |
|---------|------|--------|--------------|----------------|-------------|
| 0.60    | 10427.34 | 8.56   | 4.08         | 180.0296      | 0.003       |
| 0.73    | 440464  | 2.08   | 0.97         | 137.0015      | 0.050       |
| 0.94    | 59423480| 27.74  | 12.96        | 137.0015      | 0.400       |
| 3.73    | 4195115 | 19.57  | 9.14         | 185.1347      | 0.230       |
| 4.05    | 2493772 | 1.35   | 0.54         | 149.9227      | 0.090       |
| 4.31    | 20340047| 13.69  | 6.49         | 185.1350      | 0.130       |
| 4.65    | 21426651| 100.00 | 46.71        | 344.1657      | 0.220       |
| 5.32    | 30191750| 17.82  | 8.32         | 755.6378      | 0.140       |
| 5.25    | 27300386| 12.74  | 5.95         | 938.7991      | 0.190       |
| 6.29    | 23040331| 10.75  | 5.02         | 172.0774      | 0.400       |

| Compound | RT (min) | Observed mass (m/z) | Neutral observed mass (Da) | Theoretical mass (Da) | Mass error (ppm) | Isotope match score (%) |
|----------|----------|---------------------|---------------------------|----------------------|-----------------|------------------------|
| C14H12O3 | 4.52     | 279.0860            | 278.0779                  | 278.0786             | -3.44           | 99.11                  |

Mass errors of between -3.00 and 3.00 ppm with isotope match scores above 90% are considered confirmation of molecular formula.
**Figure S9.** LC-MS of LW-OTf + O$_2^-$ + ONOO$^-$, showing cleavage of LW-OTf to the related phenol and C=C linker oxidative cleavage.
Walkup MS Report

Data File: nr+superoxide+oneo_Pos_Smine_MS_00688.d  Sample Name: nr+superoxide+oneo
Sample Type: Sample  Position: P1-A2
Instrument Name: 6545 QToF  User Name: Xu Tian
Acq Method: Pos_Smine_MS.m  Acquired Time: 1/13/2020 3:15:29 PM
IRM Calibration Status: DA Method  DA Method: Pos_Smine_MS.m

Sample Group  Info.
Walkup Sample  Walkup Method: Pos_Smine_C18
Description  Description: C18 column chromatography

Stream Name  LC 1  Acquisition SW Version: ESI BPC[+4]; Scan Frag=125.0V nr+superoxide+oneo_Pos_Smine_MS_00688.d Smooth

Figure 1: Base peak chromatogram

User Chromatogram Peak List

| RT (min) | Area | Area % | Area Sum (%) | Base Peak (m/z) | Width (min) |
|---------|------|--------|--------------|----------------|-------------|
| 0.60    | 21955304 | 10.64  | 4.03         | 101.00305     | 0.080      |
| 0.73    | 2048328  | 9.91   | 5.62         | 101.00305     | 0.210      |
| 1.19    | 5875997  | 2.85   | 1.61         | 157.03555     | 0.270      |
| 3.68    | 39881775 | 16.90  | 18.53        | 302.34094     | 0.140      |
| 4.03    | 4706206  | 2.28   | 1.29         | 149.95737     | 0.100      |
| 4.28    | 27626340 | 13.39  | 7.59         | 185.11625     | 0.140      |
| 4.69    | 20628342 | 100.00 | 56.67        | 344.1897      | 0.200      |
| 5.18    | 23696361 | 10.51  | 5.96         | 755.6624      | 0.150      |
| 5.28    | 8012793  | 4.18   | 2.37         | 362.1901      | 0.140      |
| 5.62    | 11956274 | 5.80   | 3.29         | 419.3192      | 0.230      |

Compound Table

| Compound Label | RT (min) | Observed mass (m/z) | Neutral observed mass (Da) | Theoretical mass (Da) | Mass error (ppm) | Isotope match score (%) |
|----------------|----------|---------------------|----------------------------|-----------------------|------------------|------------------------|
| Cpd 1: C13H17N O | 4.73     | 204.1388            | 203.1324                   | 203.1310              | 1.95             | 97.95                  |

Mass errors of between -5.00 and 5.00 ppm with isotope match scores above 60% are considered confirmation of molecular formulas.
Figure S10. LC-MS of LW-OTf + O$_2^-$ + ONOO$^-$, showing C=C linker oxidative cleavage.
Scheme S2. Proposed mechanism for the reaction of LW-OTf with ONOO⁻.

Probe LW-OTf (4.8 mM, in DMSO) was diluted to 19 μM in methanol (Figure S11), then ONOO⁻ (95 μM) was added (Figure S12).
Walkup MS Report

Data File: mcsendor 20um_Pos_LoopInjection_MS_11354.d
Sample Name: mcsendor 20um
Sample Type: Sample
Instrument Name: 6545 QToF
Acq Method: Pos_LoopInjection_MS.m
IRM Calibration Status: Good
DA Method: Pos_LoopInjection_MS.m
Comment: 06/04/2020 14:58:53

Sample Group: mcsendor 20um_Pos_LoopInjection_MS
Walkup Sample: Pos_LoopInjection_MS
Description: C29H29F3MO6S
Wallup Method: Positive mode ionization using loop injection with common organic molecule isotope model
Description: LC 1
Acquisition SW Version: 6200 series TOF/MS0 series Q-TOF B.01.00 (800444.0)

Figure 1: Base peak chromatogram

User Chromatogram Peak List

| RT (min) | Area | Area % | Area Sum (%) | Base Peak (m/z) | Width (min) |
|---------|------|--------|--------------|----------------|-------------|
| 0.74    | 1984180.75 | 100.00 | 94.51 | 544.4732 | 0.152 |
| 1.21    | 1552971 | 5.81 | 5.49 | 101.0013 | 0.277 |

Compound Table

| Compound Label | RT (min) | Observed mass (m/z) | Neutral observed mass (Da) | Theoretical mass (Da) | Mass error (ppm) | Isotope match score (%) |
|---------------|----------|---------------------|---------------------------|----------------------|-----------------|-------------------------|
| Cpt 1         | 0.74     | 544.1778            | 544.1783                  | 544.1769             | 2.50            | 97.92                   |

Mass errors between ±5.00 and ±5.00 ppm with isotope match scores above 60% are considered confirmation of molecular formulas
Figure S11. HRMS of probe LW-OTf.
Walkup MS Report

Data File: SENSOR+ Seq ONCOG_Pos_Smine_MS_11357.d
Sample Name: SENSOR+ Seq ONCOG
Sample Type: Sample
Position: PI-87
Instrument Name: 6545 QToF
User Name: Xue Tan
Acq Method: Pos_Smine_MS.m
Acquired Time: 06/08/2020 15:22:05
IRM Calibration Status: DA Method: Pos_Smine_MS.m

Sample Group: ""
Wakup Sample: ""
Info: ""
Description: ""
Formula: C18H13F3O5S,C13H17NO
Wakup Method: Positive mode ionization using C18 column chromatography

Stream Name: LC 1
Acquisition SW Version: 6200 series TOF/6500 series
Q-TOP 0.09.00 (010944.0)

Figure 1: Base peak chromatogram

User Chromatogram Peak List

| RT (min) | Area | Area % | Area Sum (%) | Base Peak (m/z) | Width (min) |
|---------|------|--------|--------------|----------------|-------------|
| 0.64    | 13622576 | 64.63  | 18.67        | 101.0032       | 0.704       |
| 1.49    | 3315075  | 15.79  | 8.46         | 101.0006       | 0.153       |
| 1.72    | 2996271  | 1.42   | 0.41         | 101.0002       | 0.195       |
| 3.67    | 28491148 | 13.52  | 3.95         | 202.1285       | 0.133       |
| 4.26    | 24169124 | 11.47  | 3.31         | 185.1144       | 0.184       |
| 4.48    | 27444288 | 13.02  | 3.76         | 182.1346       | 0.119       |
| 4.71    | 10145215 | 48.13  | 13.91        | 194.1761       | 0.206       |
| 4.95    | 19124055 | 90.73  | 26.21        | 441.2970       | 0.387       |
| 5.48    | 21508150 | 100.00 | 28.89        | 419.2470       | 0.399       |
| 6.13    | 3421251  | 1.63   | 0.47         | 149.9833       | 0.272       |

Compound Table

| Compound Label | RT (min) | Observed mass (m/z) | Neutral observed mass (Da) | Theoretical mass (Da) | Mass error (ppm) | Isotope match score (%) |
|----------------|----------|----------------------|----------------------------|-----------------------|-----------------|-------------------------|
| Qp1: C15 H11 F3 O S | 4.40 | 361.0257 | 360.9944 | 360.0219 | 1.14 | 98.73 |
| Qp2: C13 H12 F4 N O | 3.51 | 204.1379 | 203.1307 | 203.1310 | -1.39 | 86.45 |

Mass errors of between 5.00 and 5.00 ppm with isotope match scores above 64% are considered confirmation of molecular formulae.
Figure: Extracted ion chromatogram (EIC) of compound.

Figure: Full range view of Compound spectra and potential adducts.

Figure: Zoomed Compound spectra view
(red boxes indicating expected theoretical isotope spacing and abundance)

### Compound Isotope peak List

| m/z   | z | Abund | Formula     | Ion   |
|-------|---|-------|-------------|-------|
| 361.6357 | 1 | 69.9%  | C15H11F3O5S | M+H+  |
| 359.6389 | 1 | 69.9%  | C15H11F3O5S | M+H+  |
| 363.6486 | 1 | 74.1%  | C15H11F3O5S | M+H+  |
| 364.6365 | 1 | 29.6%  | C15H11F3O5S | M+H+  |
| 365.6313 | 1 | 11.6%  | C15H11F3O5S | M+H+  |
Figure S12. LC-MS of LW-OTf + ONOO⁻, showing C=C linker oxidative cleavage.
5. Protocols for cell culture

Human hepatocytes (HL-7702) were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in high-glucose DMEM supplemented with 10 % fetal bovine serum, 1 % penicillin and 1 % streptomycin (w v\(^{-1}\)) at 37 °C in a 5 % CO\(_2\)/95 % air MCO-15AC incubator (SANYO, Tokyo, Japan).

6. Fluorescence imaging in live cells and MTT assay

6.1 MTT assay.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were carried out to evaluate the toxicity of probe LW-OTf. Hepatocytes (10\(^6\) cells mL\(^{-1}\)) were seeded into 96-well microtiter plates with total volumes of 200 μL well\(^{-1}\). After 12 h of incubation, various concentrations of LW-OTf (0 M, 1×10\(^{-3}\) M, 1×10\(^{-4}\) M, 1×10\(^{-5}\) M, 1×10\(^{-6}\) M, 1×10\(^{-7}\) M, 1×10\(^{-8}\) M and 1×10\(^{-9}\) M) were added, and the cells were cultured for another 24 h. Subsequently, an MTT solution (20 μL, 5 mg mL\(^{-1}\), in DMEM) was added to each well. After 4 h, the MTT solution was removed, and DMSO (150 μL) was added to each well. Finally, the absorbance at 490 nm was measured using a Triturus microplate reader.

Figure S13. Cell toxicity of LW-OTf towards HL-7702 cells with an incubation time of 24 h. Error bars represent s.d.

6.2 Fluorescence imaging experiments with cells.

Living cells were detached and reseeded onto 15 mm glass-bottomed dishes 24 h before confocal imaging and two-photon (TP) fluorescence imaging. For Figure S14-S17, confocal imaging was performed on Leica SP8 high-resolution fluorescence microscope with red channel (λ\(_{ex}\)=633 nm and λ\(_{em}\)=680-780 nm) and blue channel (λ\(_{ex}\)=405 nm and λ\(_{em}\)=410-500 nm). For Figure 4, two-photon (TP) fluorescence images were obtained with a Zeiss 880 NLO microscope and the supporting software ZEN 2 lite. TP images of probe fluorescence were obtained with NIRF red channel (λ\(_{ex}\)= 633 nm and λ\(_{em}\)=635-747 nm) and TPEF blue channel (λ\(_{ex}\)=720 nm and λ\(_{em}\)=420-550 nm). For data analysis, the average fluorescence intensity per image for each set of experimental conditions was obtained by selecting the regions of interest. Each experiment was repeated five separate times with identical results. All data are expressed as the mean ± S.D.
6.2.1 Fluorescence imaging of HL-7702 cells (pre-incubated with Tiron) and treatment with either 2-ME or 2-ME/SIN-1.

Figure S14. Confocal imaging of O$_2^\bullet^-$/ONOO$^-$ levels in hepatocytes. (a) HL-7702 cells were pretreated with Tiron (10 μM) for 0.5 h, with various concentrations of 2-ME (0, 0.5, 2.0 and 3.0 μg/mL) for 0.5 h, and then exposed to probe LW-OTf (2.4 μM) for 15 min. (b) HL-7702 cells were pretreated with Tiron (10 μM) for 0.5 h,
incubated with 2-ME (3.0 μg/mL) for 0.5 h, exposed to probe LW-OTf (2.4 μM) for 15 min, and finally incubated with various concentrations of SIN-1 (0, 0.5, 2.0, and 3.0 mM) for another 0.5 h. (a, b) Red fluorescence channel for O$_2^\cdot$; $\lambda_{ex} = 633$ nm, $\lambda_{em} = 680-780$ nm; blue fluorescence channel for ONOO$^-\;: \; \lambda_{ex} = 405$ nm, $\lambda_{em} = 410-500$ nm. (c) Red and blue relative fluorescence intensity output with various doses of 2-ME in (a). The fluorescence intensity of the control group (2-ME, 0 μg/mL) is defined as 1.0. (d) Relative fluorescence intensity output with various doses of SIN-1 in (b) in the red and blue channels. The fluorescence intensity of the control group (SIN-1, 0 mM) is defined as 1.0. Note: The data are expressed as the mean ± SD. Results were confirmed in quintuplicate. Blue channels using excitation at 405 nm represent one-photon imaging.

6.2.2 Fluorescence imaging of APAP-induced injury in HL-7702 cells.

Experimental procedure for Figure 4 and Figure S15
HL-7702 cells were divided into three groups. The control group cells were stained with probe LW-OTf (2.4 μM) for 15 min. The model group cells were incubated with APAP (20 mM) for 1 h, then stained with probe LW-OTf (2.4 μM) for 15 min. To investigate injury remediation, HL-7702 cells were pretreated with BHA (500 μM) for 1 h and then incubated with APAP (20 mM) for 1 h, followed by staining with probe LW-OTf (2.4 μM) for 15 min. The cell culture medium of each group was removed, and all cells were washed with 1.0 mL of PBS three times before fluorescence imaging. For Figure 4, two-photon photographs were taken imaged by Zeiss 880 NLO microscope and the supporting software ZEN 2 lite. For Figure S15, one-photon confocal photographs were taken imaged by Leica SP8 high-resolution fluorescence microscope and Leica Application Suite X software.
Figure S15. Confocal images of APAP-induced injury with HL-7702 cells. (a, d and g) Cells were stained with probe LW-OTf (2.4 μM) for 15 min. (b, e, and h) Cells were incubated with APAP (20 mM) for 1 h, and then stained with probe LW-OTf (2.4 μM) for 15 min. (c, f and i) Cells were pretreated with BHA (500 μM) for 1 h and then incubated with APAP (20 mM) for 1 h, followed by staining with probe LW-OTf (2.4 μM) for another 15 min. (a-c) Red fluorescence channel for $O_2^\bullet-$: $\lambda_{ex} = 633$ nm, $\lambda_{em} = 680-780$ nm; (d-f) Blue fluorescence channel for ONOO$: $\lambda_{ex} = 405$ nm, $\lambda_{em} = 410-500$ nm. (g-i) Merged fluorescence channel. (j) Red relative fluorescence intensity output of a-c. (k) Blue relative fluorescence intensity output of d-f. Note: the fluorescence intensity of control group is defined as 1.0. The data are expressed as the mean ± SD. Comparable results were obtained in five independent experiments. Blue channels using excitation 405 nm represent one-photon imaging.
6.2.3 Co-localization assay of LW-OTf.

**Experimental procedure for Figure S16.** HL-7702 cells were incubated with 2-ME (2.0 μg/mL) for 0.5 h and then co-incubated with probe LW-OTf (2.4 μM) and each corresponding commercial organelle-specific dye for 15 min.

**Experimental procedure for Figure S17.** HL-7702 cells were co-incubated with 2-ME (2.0 μg/mL) and SIN-1 (2.0 mM) for 0.5 h, followed by staining with probe LW-OTf (2.4 μM) and each corresponding commercial organelle-specific dye for 15 min.
Figure S16. Confocal images of HL-7702 cells co-stained with probe LW-OTf and the corresponding commercial organelle-specific dyes. (a, d, g, and j) Fluorescence image of probe LW-OTf (2.4 μM, red channel, Ex = 633 nm, collected 680-780 nm) in cells pretreated with 2-ME (2.0 μg/mL) for 0.5h. (b) Fluorescence image of Lyso-Tracker Green (75 nM, green channel, Ex = 488 nm, collected 495-550 nm). (c) Fluorescence image of ER-Tracker Red (75 nM, green channel, Ex = 561 nm, collected 600-630 nm). (e) Fluorescence image of Golgi-Tracker Red (75 nM, green channel, Ex = 561 nm, collected 600-630 nm). (h) Fluorescence image of Mito-Tracker Green (60 nM, green channel, Ex = 488 nm, collected 495-550 nm). (c), (f), (i), (l) Overlay of (a) and (b), (d) and (e), (g) and (h), (j) and (k), respectively. Pearson’s correlation of lysosomes, endoplasmic reticulum, Golgi apparatus and mitochondria are 0.8659, 0.5132, 0.3931 and 0.3452, respectively.
Figure S17. Confocal images of HL-7702 cells co-stained with probe LW-OTf and the corresponding commercial organelle-specific dyes. (a, d, g, and j) Fluorescence image of probe (2.4 μM, blue channel, Ex = 405 nm, collected 410-480 nm) in cells co-incubated with 2-ME (2.0 μg/mL) and SIN-1 (2.0 mM) for 0.5h. (b) Fluorescence image of Lyso-Tracker Green (75 nM, green channel, Ex = 488 nm, collected 495-550 nm). (e) Fluorescence image of ER-Tracker Red (75 nM, green channel, Ex = 561 nm, collected 600-630 nm). (h) Fluorescence image of Golgi-Tracker Red (75 nM, green channel, Ex = 561 nm, collected 600-630 nm). (k) Fluorescence image of Mito-Tracker Green (60 nM, green channel, Ex = 488 nm, collected 495-550 nm). (c), (f), (i), (l) Overlay of (a) and (b), (d) and (e), (g) and (b), (j) and (k), respectively. Pearson’s correlation of lysosomes, endoplasmic reticulum, Golgi apparatus and mitochondria are 0.8962, 0.2902, 0.4155 and 0.4752, respectively. Note: blue channels using excitation 405 nm represent one-photon imaging.
7. Near-infrared fluorescence imaging of APAP-induced injury in mice

Four to six-week old male C57 mice were purchased from the laboratory animal center of Shandong University (Jinan, China). Permission for the animal experiments was obtained from the Shandong Normal University authorities.

Experimental procedure for Figure 5a and 5b
For in vivo imaging of the livers of mice with drug-induced liver injury the model group mice were intraperitoneally injected with acetaminophen APAP, (400 mg/kg and 600 mg/kg). The control group were given APAP in analgesic dosage (200 mg/kg). After 6 h, all mice were first intraperitoneally injected with anaesthetic (10% chloral hydrate, 100 μL), and 10 min later with probe LW-OTf (200 μL, 48 μM), and left for 15 minutes. All mice underwent surgical treatment to expose the liver for in vivo imaging. The images were recorded in the red channel ($\lambda_{ex/em} = 660/710$ nm).

Experimental procedure for Figure 5c and 5d
To evaluate the deep tissue penetration of probe LW-OTf, the model group mice were intraperitoneally injected with acetaminophen APAP, (400 mg/kg and 600 mg/kg). The control group were given APAP in analgesic dosage (200 mg/kg). After 6 h, the mice were first intraperitoneally injected with anaesthetic (10% chloral hydrate, 100 μL), and 10 min later with LW-OTf (200 μL, 48 μM), and left for 15 min before depilation. The images were recorded in the red channel ($\lambda_{ex/em} = 660/710$ nm).

Experimental procedure for Figure 5e and 5f
The mice were divided into three groups. The first group received an intraperitoneal injection of APAP (200 mg/kg). The second group was given an intraperitoneal injection of APAP (600 mg/kg). The third group was first given an intraperitoneal injection of 400 mg/kg NAC, and 1 h later was injected intraperitoneally with 600 mg/kg APAP. All three groups were injected peritoneally with LW-OTf (200 μL, 48 μM) after administering APAP, after intervals of 30 min, 2h and 6h The images were recorded in the red channel ($\lambda_{ex/em} = 660/710$ nm).

Experimental procedure for Figure 7a and 7b
The model group mice were intraperitoneally injected with APAP (600 mg/kg) for 6 h. The control group were given the same volume of saline (100 μL). Subsequently, all the mice were intraperitoneally injected anaesthetic (10% chloral hydrate, 100 μL) and left for 10 min. After intraperitoneal injection of LW-OTf (200 μL, 48 μM) and 15 min incubation the mice were killed and major organs were excised, collected and imaged in the red channel ($\lambda_{ex/em} = 660/710$ nm).

8. Two-photon fluorescence imaging of APAP-induced injury in mice

Experimental procedure for Figure 6
Four to six-week old male C57 mice were fasted for 12 h and divided into three groups. The model group mice were intraperitoneally injected with APAP (400 mg/kg) and APAP (600 mg/kg) for 6 h, respectively. The control group were given APAP (200 mg/kg). Subsequently, all the mice were intraperitoneally injected with anaesthetic (10% chloral hydrate, 100 μL), and 10 min later with probe LW-OTf (200 μL, 48 μM). After 15 minutes, the mice underwent surgical treatment to expose the liver for two-photon fluorescence imaging. The liver was imaged with excitation at 720 nm and the emission was collected between 397–571 nm. Two-photon 3D imaging was acquired with the z-stack mode of the microscope.

9. Hematoxylin and eosin (H&E) staining of main organs in mice

Experimental procedure for Figure 7
The model group of mice were injected intraperitoneally with APAP (600 mg/kg) for 6 h. The control group were
given the same volume of physiological saline. Subsequently, the mice were intraperitoneally injected with anaesthetic (10% chloral hydrate, 100 μL), wait 10 min. Then the mice were injected intraperitoneally with LW-OTf (200 μL, 48 μM) and left for 15 min. Subsequently, all the mice were dissected to isolate the spleen, lungs, heart, kidneys and liver. After imaging in the red channel, the isolated organs were fixed in 4% paraformaldehyde for tissue staining. The samples were dehydrated, embedded, sectioned and stained by hematoxylin and eosin. The sections were imaged with optical microscope (NIKON Eclipse ci) and the supporting software (NIKON digital sight DS-Fi2, MADE IN JAPAN).

Beginning with splenic tissue cells (Figure 7c and 7h), red and white pulp boundaries were clear, with a large quantity of white pulp, and visible cord and sinus of the red pulp, abundant lymphocytes, and normal overall structure and morphology with no obvious necrosis or bleeding, indicating no damage to the spleen. Similarly, the pulmonary tissue cells of both control and model groups (Figure 7d and 7i) appeared healthy, as indicated by the tightly arranged bronchiolar epithelial cells with no obvious degeneration, necrosis, or distinct secretions in the cavity. Moreover, the alveolar walls were thin with no significant thickening and their structure was complete; no obvious inflammatory cell infiltration was observed in pulmonary interstitium or alveoli. Cardiac myocytes of the control and model groups (Figure 7e and 7j) also appeared healthy, staining uniformly. The myocardial fibers were regularly and tightly arranged, and with no obvious degeneration or necrosis, and no obvious inflammatory cell infiltration. Renal tissue cells in both groups (Figure 7f and 7k) showed no abnormal morphology and structure of the glomerular tissues and the renal tubules were tightly arranged. No obvious degeneration, necrosis or brush border shedding was seen, nor was there any renal interstitial damage, connective tissue hyperplasia, or any obvious inflammatory cell infiltration.

**Figure S18.** Enlarged hematoxylin and eosin (H&E) staining images of Figure 7. (c-l) The hematoxylin and eosin (H&E) staining of main organs in mice. (c-g) Spleen, lung, heart, kidney, liver tissue section of control group. (h-l) Spleen, lung, heart, kidney, liver tissue section of model group with APAP (600 mg/kg)-induced liver injury. (g) The structure of the liver lobules was clear, the hepatocytes were arranged neatly. No obvious degeneration and necrosis of hepatocytes were observed. There was no obvious congestion of hepatic sinusoid. No obvious inflammatory cell infiltration was seen. (l) Compared with the control group, there were obvious liver damages in the model group. Widely congestions and hemorrhages were visible in the sinusoids of the acinar III zone (black arrow). A large amount of hepatocytes steatosis with dense vacuoles in the cytoplasm (blue arrow) were observed. Some hepatocytes with dissolved and disappeared nucleus were necrotic (green arrow). Slight inflammatory infiltration and individual inflammatory foci in the liver lobules could be seen (yellow arrow). Scale bar = 100 μm.
10. Synthesis of probe LW-OTf

Scheme S3. Synthesis of probe LW-OTf.

Synthesis of LW-OH
A mixture of resorcinol (275 mg, 2.5 mmol) and K$_2$CO$_3$ (346 mg, 2.5 mmol) in acetonitrile (10.0 mL) was stirred under a nitrogen atmosphere at room temperature for 15 min, before addition of IR-780 iodide (667 mg, 1.0 mmol) in acetonitrile (5.0 mL). The reaction mixture was heated to 50 °C for 2 h. After cooling, the solvent was removed in vacuo, and the residue was purified by silica gel chromatography (SiO$_2$, CH$_2$Cl$_2$/MeOH, 80:1 v/v) to afford LW-OH as a blue-green solid in 10% yield (56 mg). M.p. 182 °C; $^1$H NMR (500 MHz, CDCl$_3$) δ$_H$ 11.13 (bs, 1H, -OH), 8.63 (d, J = 14.9 Hz, 1H, -N=CH), 7.58-7.15 (m, 8H, ArH), 6.12 (d, J = 14.7 Hz, 1H, -N=CC=CH), 4.12 (t, J = 7.8 Hz, 2H, CH$_2$), 2.66 (t, J = 5.9 Hz, 2H, CH$_2$), 1.99-1.90 (m, 4H, 2 x CH$_2$), 1.79 (s, 6H, 2 x -N=CCC$_3$), 1.07 (t, J = 7.4 Hz, 3H, -CH$_2$CH$_3$); $^{13}$C NMR (126 MHz, CDCl$_3$) δ$_C$ 176.1, 163.8, 163.4, 157.6, 154.9, 144.9, 141.9, 141.6, 137.0, 129.0, 128.8, 126.8, 125.4, 116.9, 114.9, 111.6, 103.2, 101.5, 50.4, 46.8, 29.8, 29.1, 28.7, 21.2, 20.6, 11.9; HRMS (ESI$^+$): calc. for C$_{28}$H$_{30}$NO$_2$[M-I]$^+$ 412.2271 m/z, found 412.2271 m/z. Characterization data were consistent with previous literature reports.

Synthesis of LW-OTf
To a stirred solution of LW-OH (200 mg, 0.37 mmol) in dichloromethane (6.0 mL) was added triethylamine (180 µL, 1.32 mmol). After cooling to 0 °C, triflic anhydride (Tf$_2$O, 187 µL, 1.11 mmol) was added dropwise, and the reaction was left to stir for 30 min, before warming to room temperature and leaving to stir a further 35 min. The reaction mixture was diluted with water (20 mL) and dichloromethane (30 mL), the layers were separated, and the aqueous phase was extracted with dichloromethane (30 mL) twice more. The combined organics were concentrated to dryness in vacuo, and the residue was purified by silica gel column chromatography (SiO$_2$, CH$_2$Cl$_2$/MeOH 60:1, v/v) to afford the title compound LW-OTf as a purple solid (178 mg, 69%). M.p. 121 °C; $^1$H NMR (500 MHz, CDCl$_3$) δ$_H$ 8.61 (d, J = 15.2 Hz, 1H, -N=CH$_3$), 7.56 – 7.51 (m, 2H, ArH), 7.50 – 7.42 (m, 3H, ArH), 7.15-7.09 (m, 2H, ArH), 7.03 (s, 1H, ArH), 6.81 (d, J = 15.3 Hz, 1H, -N=CC=CH$_2$), 4.53 (t, J = 6.9 Hz, 2H, CH$_2$), 2.78 (t, J = 6.0 Hz, 2H, CH$_2$), 2.75 (t, J = 6.0 Hz, 2H, CH$_2$), 2.03-1.91 (m, 4H, 2 x CH$_2$), 1.82 (s, 6H, 2 x -N=CCCH$_3$), 1.07 (t, J = 7.4 Hz, 3H, -CH$_2$CH$_3$); $^{13}$C NMR (126 MHz, CDCl$_3$) δ$_C$ 179.6, 158.4, 153.0, 140.9, 146.4, 142.5, 141.4, 132.2, 129.6, 129.0, 128.9,
128.6, 122.6, 122.2, 120.1 (q, $J_{CF} = 320.7$ Hz, 1C, OTf), 120.1 (q, $J_{CF} = 320.7$ Hz, 1C, OTf), 118.0, 116.2, 113.9, 109.5, 108.0, 51.5, 47.9, 29.6, 28.1, 24.2, 21.8, 20.1, 11.5; $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ -72.6 (ArOTf), -78.3 (TfO$^-$); HRMS (ESI$^+$): calc. for C$_{29}$H$_{29}$F$_3$NO$_4$S [M-OTf]$^+$ 544.1764 m/z, found 544.1774 m/z.
11. NMR spectra

Figure S19. $^1$H NMR (500 MHz, CDCl$_3$) of LW-OTf.

Figure S20. $^{13}$C$_{\{1H\}}$ NMR (126 MHz, CDCl$_3$) of LW-OTf. Inset showing 2 x CF$_3$ quartets (red and blue are separate quartets).
Figure S21. $^{19}$F NMR (470 MHz, CDCl$_3$) of LW-OTf.
12. Characterisation of LW-OTf triflate salt

Upon characterization of LW-OTf following the synthesis described above, it was observed that an anion exchange had occurred, exchanging the original iodide counteranion I\(^-\) IR-780 iodide for a triflate TfO\(^-\) (by product of the triflation reaction). The experiments described below were therefore carried out in order to confirm this observation.

Two quantitative \(^{19}\)F NMR experiments were carried out initially. The first used a single scan \(^{19}\)F NMR method, producing an inherently quantitative spectrum (Figure S22). The second experiment instead used a 16-scan \(^{19}\)F NMR experiment with an increased relaxation time \(T1 = 40\) s, allowing for accurate integration of the resulting signals (Figure S23). Both experiments showed a 1:1 ratio of the \(^{19}\)F signals, indicating a 1:1 ratio of the two triflate environments, consistent with our suggested structure of salt LW-OTf, containing both a phenol triflate moiety and a triflate counteranion.

As it was acknowledged that this equimolar ratio could be coincidental and caused by residual TfOH byproduct from the previous synthetic step, further experiments were carried out (full procedure below). Addition of TfOH to a sample of LW-OTf showed an increase in intensity and broadening of the signal at -78.3 ppm by \(^{19}\)F NMR, indicating this peak to be the TfOH/TfO\(^-\) signal (Figure S24). This mixture was then washed with water, dried, and the resulting product was analyzed by quantitative \(^{19}\)F NMR spectroscopy (Figures S25 and S26). This revealed that the intensities of the triflate \(^{19}\)F signals of LW-OTf had returned to a 1:1 ratio, indicating that excess triflate had been removed by the wash, and that all remaining signal was in fact part of the complex.

Previous reports in the literature have shown that iodide anions are capable of exchanging with triflates to produce stable heteroatomic salts and quaternary ammonium salts.\(^2\) Additionally, comparison of the pKa’s of HI (-9.5 in water, -10.9 in DMSO) and TfOH (-14.7 in water, -14.3 in DMSO) indicates that the former is more likely to protonate and therefore be removed during workup.\(^3\) Moreover, comparison of their boiling points (HI: -35.4 °C; TfOH: 162 °C)\(^4\)\(^5\) shows that upon exchange, HI will quickly evaporate even at atmospheric pressure, favoring the formation of the triflate salt. These properties and reports confirm the likelihood of iodide to triflate anion exchange in our hemicyanine system.

At this stage we would like to note that a surprising number of reports of hemicyanine dyes were found in the literature that either overlooked the possibility of anion exchange or ignored the counteranion entirely, likely leading to inaccurate measurements of concentration and stoichiometry. We hope that this thorough characterization of the counter-anion will serve as an example of good practice, hopefully leading to more consistent structural determination and reporting of hemicyanine structures in the future.

Experimental procedure for doping of LW-OTf with TfOH and washing: Triflic acid (5.3 \(\mu\)L, 60 \(\mu\)mol) was added to a stirred solution of LW-OTf (20 mg, 29 \(\mu\)mol) in DCM (1.0 mL) at 0 °C. After stirring for 1 h, the solvent was removed in vacuo, and quantitative \(^{19}\)F NMR was carried out (Figures S24). The product was then re-dissolved in DCM (10 mL), washed with water (3 \(\times\) 10 mL), dried over MgSO\(_4\) and concentrated in vacuo. \(^{19}\)F NMR quantitative NMR was then taken (Figures S25 and S26).
Figure S22. $^{19}$F NMR (470 MHz, CDCl$_3$, 1 scan) of LW-OTf.

Figure S23. $^{19}$F NMR (470 MHz, CDCl$_3$, 16 scans, $T1 = 40$ s) of LW-OTf.
Figure S24. $^{19}$F NMR (470 MHz, CDCl$_3$, 16 scans, $T_I = 40$ s) of LW-OTf + TfOH.

Figure S25. $^{19}$F NMR (470 MHz, CDCl$_3$, 1 scan) of washed LW-OTf + TfOH.
Figure S26. $^{19}$F NMR (470 MHz, CDCl$_3$, 16 scans, $T1 = 40$ s) of washed LW-OTf + TfOH.
13. Fluorescence “turn on” mechanisms and selectivity of LW-OTf and other hemicyanine-xanthene dyes

As illustrated in Scheme 1 and section 4 “Mass spectroscopic analysis” above, it is our understanding that the fluorescent turn-on of probe LW-OTf can occur in two ways: 1) Cleavage of the triflyl by superoxide to generate LW-OH, and oxidative cleavage of the alkene C=C linker of LW-OH to produce LW-XTD. The absorption and fluorescence data presented above clearly support the sequential formation of a hemicyanine intermediate followed by a D−π−A xanthene fluorophore. 2) LW-OTf can be cleaved to form LW-XTD-OTf in presence of ONOO−. This species can then be triflyl-deprotected by superoxide to form LW-XTD, which unfortunately could not be confirmed by LC-MS since ONOO− is prepared in water and KO2 decomposes in water.6

Although the reaction of superoxide with aryl triflate is now a well-established process,7,8 significant debate remains as to the reaction of peroxynitrite with these types of dyes. In most cases, oxidative cleave of the linker is reported,9,10 although the exact mechanisms of addition/oxidation has yet to be established. In certain cases, oxidation of the linker is not reported (Scheme S4a and b), although these instances seem predicated on the presence of another highly peroxynitrite-reactive functionality such as the carbonate or selenide.11,12 One isolated report has suggested that the hemicyanine-xanthene dye undergoes oxidative cleavage, stopping the FRET of SiRho-HD, thus leading to FRET “turn-off”.13

![Scheme S4](image)

**Scheme S4.** Alternative fluorescent ONOO− “turn-on” mechanisms for hemicyanine dyes. (a) Oxidation-deprotection of trifluoromethyl ketone. (b) Organoselenium oxidation for PeT quenching. (c) Xanthene oxidative ring opening for FRET quenching.

14. Statistical analysis

For each experiment, unless otherwise noted, n represents the number of individual biological replicates. For data analysis of NIRF and TPEF images of cells (Fig. 4), we firstly selected regions of interest (ROIs) of 5 images of each group (n=5), then we used Zeiss software to measure fluorescence intensity and output intensity. For data analysis of one-photon fluorescence images of cells (except Fig. 4), we firstly selected ROIs of 5 images of each group (n=5), then we used Leica Application Suite X software to measure fluorescence intensity and output intensity. For data analysis of in vivo TPEF images of mice, we firstly selected ROIs of 5 images of each group (n=5, five mice in each group), then we used Zeiss software to measure fluorescence intensity and output intensity. For data analysis of in vivo NIRF images of mice, we firstly selected ROIs of 5 images of each group (n=5, five
mice in each group), then we use Living Images software to measure fluorescence intensity and output intensity. All data are expressed as the mean ± S.D. Each experiment was repeated 5 times with identical results.

15. References

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