Electronic supplementary information (ESI) for

Ratiometric fluorescent probes for capturing endogenous hypochlorous acid in the lungs of mouse †

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† Electronic supplementary information (ESI) available.
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Table of Contents

Experimental Details S2
Table S1 S10
Figure S1-S14 S11
1H NMR spectra of ClO1-ClO6 S19
References S22

S1
Experimental Details

General information

Chemicals were purchased from Sigma Aldrich, TCI, Alfa Aesar and other commercial suppliers. All the chemicals were used directly without further purification. $^1$H NMR were recorded on a Bruker AVIII400HD NMR spectrometer in CDCl$_3$. High resolution mass spectra were obtained using a Thermo LTQ Orbitrap. Absorption and fluorescence spectra were measured on a Hitachi U-2910 UV/Vis double-beam spectrophotometer and MolecularDevices Spectramax M5 plate reader.

Synthetic procedures and characterizations of ClO1-ClO6

Cy7-C18 and Cy7-C4 were synthesized according to previously reported methods$^1$: Cy7-Cl (200 mg, 0.26 mmole) and Stearylamine (200 mg, 0.74 mmole) were stirred in 5 mL DMF at 70 °C. The reaction was quenched by adding 50mL CH$_2$Cl$_2$, when TLC showed complete reaction. The organic phase was washed with 20 mL saturated brine for twice. The organic phase was collected and dried with magnesium sulfate anhydrous. After evaporation, the rough product was purified through CombiFlash RF system using a RediSep Gold Resolution silica column with elution CH$_2$Cl$_2$/MeOH (100/0-90/10 by volume), yielding 143 mg (yield 55%) dark blue solid. Cal. M$^+$: C$_{60}$H$_{90}$N$_3$O$_4^+$, 916.6926; found: 916.6904.

Cy7-Cl (200 mg, 0.26 mmole) and n-butylamine (200 µL, 2.0 mmole) were stirred in 5 mL DMF at 70 °C. The reaction was quenched by adding 50mL CH$_2$Cl$_2$, when TLC showed complete reaction. The organic
phase was washed with 20 mL saturated brine twice. The organic phase was collected and dried with Magnesium sulfate anhydrous. After evaporation, the rough product was purified through CombiFlash Rf system using a RediSep Gold Resolution silica column with elution CH₃Cl₂/MeOH (100/0-90/10 by volume), yielding 133 mg (yield 63%) dark blue solid. Cal. M⁺: C₄₆H₆₂N₃O₄⁺, 720.4735; found: 720.4717.

Synthesis of ClO1: Cy7-C18 (100 mg, 0.10 mmole) and [2-[2-(Dimethylamino)ethyl]methylamino]ethanol (100µL, 0.62mmole) were dissolved in 10 mL CH₂Cl₂, followed by adding EDC (50mg, 0.26 mmole) and DMAP (10mg). The reacting mixture was stirred at RT overnight. The organic phase was washed with 10 mL saturated brine twice. The organic phase was collected and dried with Magnesium sulfate anhydrous. After evaporation, the rough product was purified through CombiFlash Rf system using a RediSep Gold Resolution silica column with elution CH₂Cl₂/ultra (100/0-50/50 by volume), yielding 40 mg (yield 32%) dark blue solid. Ultra: CH₂Cl₂/MeOH/NH₄OH = 75/22/3 by volume. Cal. M⁺: C₇₄H₁₂₂N₇O₄⁺, 1172.9553, found: 1172.9550. ¹H NMR (400 MHz, CDCl₃) δ = 7.72-7.69 (1H, d, J = 12), 7.24-7.20 (4H, t, J = 8), 7.01-6.98 (2H, t, J = 8), 6.77-6.75 (2H, d, J = 8), 5.52-5.49 (2H, d, J = 12), 4.19-4.16 (3H, t, J = 8), 3.87 (2H, s), 3.74-3.71 (4H, t, J = 8), 2.67-2.64 (3H, t, J = 8), 2.55-2.46 (8H, m), 2.41-2.24 (24H, m), 2.00 (2H, s), 1.71 (22H, s), 1.47-1.45 (5H, m), 1.23 (31H, s), 0.89-0.85 (3H, t, J = 8).
Synthesis of ClO2: Cy7-C18 (100 mg, 0.10 mmole) and 3-Dimethylamino-1-propanol (50 µL, 0.43 mmole) were dissolved in 10 mL CH₂Cl₂, followed by adding EDC (50 mg, 0.26 mmole) and DMAP (10 mg). The reacting mixture was stirred at RT overnight. The organic phase was washed with 10 mL saturated brine twice. The organic phase was collected and dried with Magnesium sulfate anhydrous. After evaporation, the rough product was purified through CombiFlash Rf system using a RediSep Gold Resolution silica column with elution CH₂Cl₂/ultra (100/0-70/30 by volume), yielding 48 mg (yield 41%) dark blue solid. Cal. M⁺: C₇₀H₁₁₂N₅O₄⁺, 1086.8709, found: 1086.8666. ¹H NMR (400 MHz, CDCl₃) δ = 7.74-7.71 (1H, d, J = 12), 7.26-7.22 (3H, t, J = 8), 7.04-7.00 (2H, t, J = 8), 6.79-6.77 (2H, d, J = 8), 5.54-5.51 (2H, d, J = 12), 4.14-4.11 (3H, t, J = 8), 3.89-3.85 (2H, d, J = 8), 3.76-3.73 (3H, t, J = 8), 3.25-3.16 (2H, m), 2.50-2.41 (6H, m), 2.36-2.32 (8H, m), 2.26 (4H, s), 2.21 (10H, s), 2.02 (2H, s), 1.83-1.26 (24H, m), 1.48-1.47 (5H, m), 1.31-1.24 (29H, m), 0.90-0.86 (3H, t, J = 8).

Synthesis of ClO3: ClO2 (20 mg, 0.02 mmole) was dissolved in 1 mL anhydride CH₂Cl₂ followed by adding CH₃I (100 µL, 1.61 mmole). The reacting mixture was stirred at RT for 1 h. The reaction was quenched by adding 5 mL of ethyl ether. An oil like dark blue solid was obtained after centrifuge, which was treated with 2 mL of ethyl ether one more time. Cal. M³⁺: C₇₂H₁₁₈N₅O₄³⁺, 372.3056, found: 372.3065. ¹H NMR (400 MHz, CDCl₃) δ = 7.74-7.71 (1H, d, J = 12), 7.26-7.22 (3H, t, J = 8), 7.04-7.00 (2H, t, J = 8), 6.79-6.77 (2H, d, J = 8), 5.54-5.51 (2H, d, J = 12), 4.14-4.11 (3H, t, J = 8), 3.89-3.85 (2H, d, J = 8), 3.76-3.73 (3H, t, J = 8), 3.25-3.16 (2H, m), 2.50-2.41 (6H, m), 2.36-2.32 (8H, m), 2.26 (4H, s), 2.21 (10H, s), 2.02 (2H, s), 1.83-1.26 (24H, m), 1.48-1.47 (5H, m), 1.31-1.24 (29H, m), 0.90-0.86 (3H, t, J = 8).
Synthesis of ClO₄: Cy7-C18 (100 mg, 0.10 mmole) and 2-(hexamethyleneimino)ethanol (50µL, 0.37mmole) were dissolved in 10 mL CH₂Cl₂, followed by adding EDC (50mg, 0.26 mmole) and DMAP (10mg). The reacting mixture was stirred at RT overnight. The organic phase was washed with 10 mL saturated brine twice. The organic phase was collected and dried with Magnesium sulfate anhydrous. After evaporation, the rough product was purified through CombiFlash Rf system using a RediSep Gold Resolution silica column with elution CH₂Cl₂/ultra (100/0-70/30 by volume), yielding 50 mg (yield 40%) dark blue solid. Cal. M⁺: C₇₆H₁₂₀N₅O₄⁺, 1166.9335, found: 1166.9314. ¹H NMR (400 MHz, CDCl₃) δ = 7.80-7.77 (1H, d, J = 12), 7.40-7.31 (3H, m), 7.13-7.08 (3H, m), 5.88-5.84 (1H, d, J = 16), 5.51 (4H, s), 4.21-4.18 (3H, t, J = 8), 4.00-3.97 (2H, t, J = 8), 3.81-3.78 (2H, d, J = 8), 3.54-3.48 (6H, m), 3.40-3.37 (5H, m), 3.20 (10H, s), 3.16-3.14 (20H, s), 2.91 (3H, s), 2.58 (2H, s), 2.54-2.41 (3H, t, J = 8), 2.18 (3H, s), 2.01-1.97 (5H, m), 1.91-1.81 (5H, m), 1.76-1.69 (11H, m), 1.28 (22H, s), 1.22-1.18 (4H, t, J = 8), 1.14-1.10 (8H, t, J = 8), 0.93-0.90 (3H, t, J = 8).
Synthesis of **ClO5**: **Cy7-C18** (100 mg, 0.10 mmole) and 1-butanol (50µL, 0.55mmole) were dissolved in 10 mL CH₂Cl₂, followed by adding EDC (50mg, 0.26 mmole) and DMAP (10mg). The reacting mixture was stirred at RT overnight. The organic phase was washed with 10 mL saturated brine twice. The organic phase was collected and dried with Magnesium sulfate anhydrous. After evaporation, the rough product was purified through CombiFlash Rf system using a RediSep Gold Resolution silica column with elution CH₂Cl₂/methanol (100/0-95/5 by volume), yielding 65 mg (yield 51%) dark blue solid. Cal. M⁺: C₆₀H₁₀₆N₃O₄⁺, 1028.8178, found: 1028.8179. ¹H NMR (400 MHz, CDCl₃) δ = 7.74-7.71 (1H, d, J = 12), 7.29-7.25 (3H, m), 7.08-7.04 (2H, m), 6.85-6.83 (1H, d, J = 8), 5.61-5.58 (1H, d, J = 12), 4.10-4.07 (4H, t, J = 8), 3.89-3.81 (5H, m), 2.50-2.47 (6H, m), 2.36-2.33 (4H, m), 1.98 (2H, s), 1.85-1.58 (23H, m), 1.50-1.49 (4H, m), 1.39-1.25 (38H, m), 0.96-0.87 (9H, m).

Synthesis of **ClO6**: **Cy7-C4** (100 mg, 0.12 mmole) and 3-Dimethylamino-1-propanol (50µL, 0.43mmole) were dissolved in 10 mL CH₂Cl₂, followed by adding EDC (50mg, 0.26 mmole) and DMAP (10mg). The reacting mixture was stirred at RT overnight. The organic phase was washed with 10 mL saturated brine twice. The organic phase was collected and dried with Magnesium sulfate anhydrous. After evaporation, the rough product was purified through CombiFlash Rf system using a RediSep Gold Resolution silica column with elution CH₂Cl₂/ultra (100/0-70/30 by volume), yielding 50 mg (yield 41%) dark blue solid. Cal. M⁺: C₅₆H₈₄N₅O₄⁺, 890.6518, found: 890.6499. ¹H NMR (400 MHz, CDCl₃) δ = 7.72-7.70 (1H, d, J = 8), 7.29-7.25 (3H, m), 7.08-7.04 (2H, m), 6.85-6.83 (1H, d, J = 8), 5.61-5.58 (1H, d, J = 12), 4.10-4.07 (4H, t, J = 8), 3.89-3.81 (5H, m), 2.50-2.47 (6H, m), 2.36-2.33 (4H, m), 1.98 (2H, s), 1.85-1.58 (23H, m), 1.50-1.49 (4H, m), 1.39-1.25 (38H, m), 0.96-0.87 (9H, m).
7.24 (1H, m), 7.05-7.02 (1H, m), 6.81-6.79 (1H, d, J = 8), 5.56-5.52 (1H, d, J = 16), 4.15-4.12 (1H, t, J = 8), 3.86-3.73 (2H, m), 3.26-3.17 (16, m), 2.51-2.48 (2H, m), 2.42-2.39 (8H, m), 2.25-2.24 (27H, m), 1.82-1.77 (4H, m), 1.70-1.66 (12H, m), 1.17-1.13 (12H, t, J = 8), 0.96-0.87 (3H, m).
Absorption and emission spectra of ClO1-ClO6 towards HClO

Stock solution of probes were prepared in DMF. All the samples were measured in phosphate buffered saline (PBS) buffer (pH 7.4) containing SDS (10 mg/mL) at room temperature. NaClO solutions with different concentrations were prepared by serial dilution of commercial NaClO solution in PBS and the concentration of the ClO\(^{-}\) stock solution was determined by measuring the absorbance at 290 nm with a molar extinction coefficient of 350 M\(^{-1}\)cm\(^{-1}\). The absorption and emission spectra of ClO1-ClO6 in PBS by using the NaClO solutions as the HClO source.

Detection limit of ClO1 towards HClO

The fluorescence spectrum of ClO1 was measured three times and the standard deviation of the fluorescence ratio of blank measurement was obtained. The fluorescence ratio (I\(_{605}\)/I\(_{760}\)) was plotted against the concentration of NaClO. The detection limit was calculated through the equation 3\(\sigma/k\), where \(\sigma\) is the standard deviation of fluorescence ratio of blank measurement, \(k\) is the slope between the fluorescence ratio and NaClO concentration.

Selectivity of ClO1 towards HClO over other ROS/RNS, metal ions and amino acids

The selectivity of ClO1 towards HClO was studied by comparing the intensity of absorption maximum (638 nm) and fluorescence at 760 nm of ClO1 (5 µM) upon the addition of NaClO and other ROS/RNS, metal ions, amino acids. Absorption spectra and fluorescence spectra of ClO1 (5 µM) with or without 10 µM of NaClO, 50 µM of other ROS/RNS, 50 µM of metal ions or 10 µM of amino acids were measured in phosphate buffered saline (PBS) buffer (pH 7.4) containing SDS (10 mg/mL) at room temperature. In addition, the absorption response at 638 nm and fluorescence response at 760 nm of ClO1 (5 µM) towards 10 µM of NaClO in the presence of 50 µM of Fe\(^{2+}\) (column 23), 50 µM of H\(_2\)O\(_2\) (column 24) and 50 µM of Glutathione was also studied. The intensity of absorption maximum at 638 nm and fluorescence at 760 nm was normalized with that of control group.

In vitro ratiometric fluorescence imaging of exogenous and endogenous HClO

For the imaging of exogenous HClO, A549 cells were seeded in 6-well clear bottom plate with a coverslip (2 × 10\(^5\) cells/2 mL). After 24 h incubation, 2 µL of 5 mM probe stock was added into the well. After 15 min of treatment, cells were washed twice before used for imaging or NaClO stimulation. For treated group, stained cells were further treated with 50 µM NaClO for 10 min before imaging. Fluorescence imaging was performed on Olympus F1000 confocal microscope. 540 nm laser and 560-660 nm collecting filter were used for green channel; 635 nm laser and 655-755 nm collecting filter were used for red channel.

For the imaging of endogenous HClO, A549 cells were seeded in 6-well clear bottom plate with a coverslip (2 × 10\(^5\) cells/2 mL). After 24 h incubation, 2 µL of 5 mM probe stock was added into the well. After 15 min of treatment, cells were washed with PBS for twice and further treated with 500 µM lipopolysaccharide
(LPS) for 24 h before imaging. Fluorescence imaging was performed on Olympus F1000 confocal microscope. 540 nm laser and 560-660 nm collecting filter were used for green channel; 635 nm laser and 655-755 nm collecting filter were used for red channel.

**In vitro cytotoxicity of ClO1**

To evaluate the cytotoxicity of ClO1, an MTT assay was performed on A549 cells. A549 cells were seeded in a 96-well plate at the density of $5 \times 10^3$ cells per well. After 24 h of incubation, cells were treated with ClO1 at a sequence of concentrations from 0.5 µM to 10 µM. After 24 h of treatment, cell viability of each treated groups was evaluated using MTT assay as previously reported and normalized by untreated cells. The absorbance at the wavelength of 570 nm was measured on a SpectraMax M5 microplate reader (Molecular Devices, LLC., Sunnyvale, CA).

**Organ distribution of ClO1-ClO6 in mice**

All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of The Ohio State University and Experiments were approved by the Animal Ethics Committee of Institutional Animal Care and Use Committee (IACUC). For organ distribution study, all six probes ClO1-ClO6 and ICG (dissolved in PBS with 10% DMSO) were intravenously injected into of C57BL/6 mice with triple-replication at the dose of 0.56 µmole/kg. 1 h after the injection, organs of mice were harvested and imaged with Optical imaging system IVIS Lumina II. 640 nm laser and 695-770 nm emission filter were used. The Radiance efficiency of organs was normalized by the weight of each organ.

**Ratiometric fluorescence imaging of ClO1 in LPS-induced lung inflammation mouse model**

All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of The Ohio State University and Experiments were approved by the Animal Ethics Committee of Institutional Animal Care and Use Committee (IACUC). For study of ClO1 in the LPS-induced lung inflammation mouse model, C57BL/6 mice were given LPS (0.4mg in 40 µL PBS) through intranasal administration. The mice were kept for 24 h, following intravenous injection of ClO1 (0.56 µmole/kg in PBS). Control mice were given intravenous injection of ClO1 (0.56 µmole/kg in PBS) without pre-treated with LPS. 1 hour after the injection, lungs of both mice were harvested and sectioned into ~100 µm slices. The lung slices were used for fluorescence imaging on Olympus confocal microscope after sealed in coverslip. 540 nm laser and 560-660 nm collecting filter were used for green channel; 635 nm laser and 655-755 nm collecting filter were used for red channel.
Table S1. Properties of recent fluorescent HClO probes.

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<td>mouse</td>
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<td>RHSDN⁴</td>
<td>406/530-590</td>
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<td>--</td>
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<td>XWJ⁵</td>
<td>370/550-670</td>
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<td>365/374-477</td>
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<td>D. magna and zebrafish</td>
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Figure S1. Mass spectra of ClO1 after treated with NaClO solution.
Figure S2. Absorption spectra of 5.0 µM ClO1 treated with various concentrations of NaClO (0-14 µM).

Figure S3. A linear correlation between the fluorescence ratios ($I_{605}/I_{760}$) of ClO1 and the concentration of NaClO. The equation is $y = 0.1178x + 0.0814$ with $R^2 = 0.9546$. 

Figure S6. Absorption (A) and emission (B) spectra of 5.0 µM ClO2 treated with various concentration of NaClO (0-8 µM). Inset is the fluorescence ratio ($I_{605}/I_{760}$) change against the concentration of NaClO.

Figure S7. Absorption (A) and emission (B) spectra of 5.0 µM ClO3 treated with various concentration of NaClO (0-9 µM). Inset is the fluorescence ratio ($I_{605}/I_{760}$) change against the concentration of NaClO.
Figure S8. Absorption (A) and emission (B) spectra of 5.0 µM ClO$_4^-$ treated with various concentration of NaClO (0-14 µM). Inset is the fluorescence ratio ($I_{605}/I_{760}$) change against the concentration of NaClO.

Figure S9. Absorption (A) and emission (B) spectra of 5.0 µM ClO$_5^-$ treated with various concentration of NaClO (0-14 µM). Inset is the fluorescence ratio ($I_{605}/I_{760}$) change against the concentration of NaClO.
Figure S10. Absorption (A) and emission (B) spectra of 5.0 µM ClO6 treated with various concentration of NaClO (0-14 µM). Inset is the fluorescence ratio (I_{605}/I_{760}) change against the concentration of NaClO.

Figure S11. Fluorescence imaging of exogenous HClO in A549 cells. Cells were imaged with ClO2 in the absence (A-C) or presence of (D-F) NaClO. A and D are green channel (560-660 nm); B and E are red channel (655-755 nm); C and F are ratio image of green/red channel, color bar 0-5. Scale bar 20 µM.
Figure S12. Fluorescence imaging of exogenous HClO in A549 cells. Cells were imaged with ClO4 in the absence (A-C) or presence of (D-F) NaClO. A and D are green channel (560-660 nm); B and E are red channel (655-755 nm); C and F are ratio image of green/red channel, color bar 0-5. Scale bar 20 µM.
Figure S13. Fluorescence imaging of exogenous HClO in A549 cells. Cells were imaged with ClO6 in the absence (A-C) or presence of (D-F) NaClO. A and D are green channel (560-660 nm); B and E are red channel (655-755 nm); C and F are ratio image of green/red channel, color bar 0-5. Scale bar 20 µM.

Figure S14. Survive rate of A549 cells after incubated with various concentrations of ClO1 for 24 h.
$^1$H NMR spectra of ClO1-ClO6

$^1$H NMR spectra of ClO1

$^1$H NMR spectra of ClO2
$^1$H NMR spectra of ClO$_3$^−

$^1$H NMR spectra of ClO$_4$^−
$^1$H NMR spectra of ClO₅

$^1$H NMR spectra of ClO₆
References