Supporting information

A fast-responsive mitochondria-targeted fluorescent probe detecting endogenous hypochlorite in living RAW 264.7 cells and nude mouse

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Experiment

Equipments

$^1$H NMR and $^{13}$C NMR spectra were recorded on Bruker Ultrashield 300 MHz NMR spectrometer. Chemical shifts were expressed in ppm (in DMSO-d$_6$; TMS as internal standard) and coupling constants ($J$) in Hz. Mass spectroscopy was obtained from SHIMADZU LCMS-2020 and Agilent Technologies 6540 UHD Accurate-Mass Q-TOF LC/MS. Fluorescence spectra were measured using Hitachi Fluorescence spectrophotometer-F-4600. Absorption spectra were measured on a Perkin Elmer Lambda 35 UV/VIS spectrophotometer. Fluorescent images were captured by Olympus FV-1000 laser scanning confocal fluorescence microscope. Imaging of living mouse was conducted employing Perkin Elmer IVIS Lumina Spectrum Imaging System.

Cell culture

RAW 264.7 macrophage cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum and antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin), maintaining at 37 °C in a humidified atmosphere of 95% air and 5% CO$_2$.

Cell imaging

Fresh stock of RAW 264.7 macrophage cells was seeded into a glass bottom dish with a density of $1\times10^5$ cells per dish, and incubated for 24 h. Subsequently, the cells were exposed to 5 μM PZ–Py solution for 10 min at room temperature. The solution was then removed, and the cells were washed with PBS (2 mL×3) to clear PZ–Py molecules attached to the surface of cells. In the control experiment, after the macrophage cells have been incubated with LPS (1 μg/mL) for 5 h, and then further coincubated with PMA (1μg/mL) and PZ–Py (5 μM) for 20 min. The culture medium was removed, and the treated cells were washed three times with PBS (2 mL×3) before observation. Fluorescence imaging was performed with confocal laser scanning microscopy (Olympus, FV-1000; λ$_{ex}$=405 nm; fluorescent signals were collected at 530–630 nm). The images were captured using a photomultiplier.

Fluorescence Imaging in Living Mouse

A nude mouse (20–25 g) was given a skin-pop injection of LPS (100 μL×1 μg/mL). After 12 h, PMA was injected to the same region. 30 min later, the mouse was anesthetized by inhalation of isoflurane. Then a solution of the probe PZ–Py (50 μM×50 μL in saline, containing 1% DMSO) was injected to the same region. As a control, unstimulated mouse given a skin-pop injection only with the probe PZ–Py (20 μM×50 μL in saline, containing 1% DMSO) were also prepared. The pictures were taken after the mouse was incubated for 0, 5, 10, 15, 20, 30, 45 and 60 min, respectively. All experiments were performed in compliance with the Regulations for the Administration of Affairs Concerning Experimental Animals published by Bureau of Legislative Affairs of the State Council of the People's Republic of China and guidelines of State Key Laboratory of Pharmaceutical Biotechnology in Nanjing University, and in addition, the institutional committee(s) approved the experiments.
Synthesis

Preparation and Characterization of 2

To a 250-mL three-necked round-bottom flask containing N-methylformanilide (1.62 g, 12 mmol) was added phosphorus oxychloride (2.30 g, 15 mmol) by dropwise. The mixture was stirred at room temperature under N\textsubscript{2} for 30 min. Then the mixture was added to a solution of 1 (2.13 g, 10 mmol) in 20 mL 1,2-dichloroethane by dropwise, and the reaction mixture was stirred at 85 °C under N\textsubscript{2} atmosphere for 6 h. After cooling to the room temperature, the mixture was poured into ice water slowly, and then neutralized with 0.1 M NaOH. Afterwards, the mixture was extracted with dichloromethane (3×50 mL). The extractions were combined, washed with 0.1 M HCl, distilled water, saturated brine, and then dried over anhydrous sodium sulfate. After evaporation of solvent, the crude compound was purified over silica gel column and desired compound was eluted by PE: EA (10:1 v/v) to obtain 1.81 g pure product as yellow solid. Yield: 75%. ESI-MS: m/z 242.00 [M+H]\textsuperscript{+} (calcd. 242.06).

Preparation and Characterization of 3

4-picoline (1.86 g, 20 mmol) and methyl iodide (3.55 g, 25 mmol) were mixed in toluene. The solution was stirred at room temperature overnight. After completion of the reaction, the solution was filtered. The solid was washed with ethyl ether and was further dried under vacuum to afford 3 (4.19 g, 41 mmol) as a light yellow solid. Yield: 89%. \textsuperscript{1}H NMR (400 MHz, DMSO-d\textsubscript{6}) \(\delta\) 8.82 (d, \(J\) = 6.0 Hz, 2H), 7.95 (d, \(J\) = 6.0 Hz, 2H), 4.27 (s, 3H), 2.58 (s, 3H). ESI-MS: m/z 108.12 [M-I]\textsuperscript{-} (calcd. 108.08).

Preparation and Characterization of PZ–Py

Compound 2 (241 mg, 1 mmol), 3 (258 mg, 1.1 mmol) and 0.5 mL piperidine were mixed in 10 mL anhydrous ethanol. The mixture was heated to reflux overnight and was then cooled to room temperature. The precipitate was filtered off, washed by cold ethanol and was further dried under vacuum to afford PZ–Py (376 mg, 0.82 mmol) as a dark green solid. Yield: 82%. HR-MS: m/z 331.1267 [M+H]\textsuperscript{+} (calcd. 331.1269). \textsuperscript{1}H NMR (300 MHz, DMSO-d\textsubscript{6}) \(\delta\) 8.79 (d, \(J\) = 6.8 Hz, 1H), 8.11 (d, \(J\) = 6.8 Hz, 1H), 7.90 (d, \(J\) = 16.3 Hz, 1H), 7.56 m, 1H), 7.39 (d, \(J\) = 16.3 Hz, 1H), 7.28 –
7.13 (m, 1H), 7.09 – 6.92 (m, 1H), 4.22 (s, 1H), 3.36 (s, 1H). \(^{13}\)C NMR (75 MHz, DMSO-d\(_6\)) \(\delta\) 153.10, 147.45, 145.30 (2C), 144.67, 140.05, 130.08, 129.36, 128.47, 127.35, 126.16, 124.64, 123.51 (2C), 123.07, 121.69, 121.61, 115.55, 115.35, 47.28, 35.97.

**Characterization of the product of PZ–Py oxidized by NaClO**

HR-MS: \(\text{m/z 347.1215} \ [\text{M+H}]^+ \) (calcd. 347.1218). \(^1\)H NMR (300 MHz, DMSO) \(\delta\) 8.83 (d, \(J = 6.7\) Hz, 1H), 8.34 (d, \(J = 1.8\) Hz, 1H), 8.17 (d, \(J = 6.8\) Hz, 1H), 8.13 – 7.97 (m, 1H), 7.82 – 7.70 (m, 1H), 7.67 (d, \(J = 8.1\) Hz, 1H), 7.58 (d, \(J = 16.4\) Hz, 1H), 7.35 (t, \(J = 7.3\) Hz, 1H), 4.24 (s, 1H), 3.81 (s, 1H). \(^{13}\)C NMR (75 MHz, DMSO-d\(_6\)) \(\delta\) 152.92, 145.42 (2C), 140.64, 139.41, 139.25, 133.67, 132.49, 131.43, 131.25, 129.15, 124.92, 124.67, 123.69 (2C), 122.93, 122.59, 117.51, 117.09, 47.38, 36.05.
Fig. S1 Absorption spectra of PZ–Py (100 μM) upon addition of various ROS in PBS (pH 7.3, 10 mM, containing 1 % DMSO). (1: blank, 2: ClO⁻, 3: ·OH, 4: ONOO⁻, 5: O₂⁻, 6: tBuOOH, 7: H₂O₂, 8: NO). [ClO⁻] = 1 mM, [·OH] = [ONOO⁻] = [O₂⁻] = [tBuOOH] = [H₂O₂] = [NO] = 10 mM.

Fig. S2 Fluorescence intensity of PZ–Py (5 μM) at 562 nm in the absence or presence of various nucleophiles. λ_{ex} = 400 nm. 1: blank; 2: ClO⁻; 3: {HSO}_3⁻; 4: SCN⁻; 5: S²⁻; 6: CN⁻; 7: F⁻; 8: GSH; 9: Cys; 10: Hcy. [ClO⁻] = 50 μM; [{HSO}_3⁻] = [SCN⁻] = [S²⁻] = [CN⁻] = [F⁻] = 500 μM; [GSH] = [Cys] = [Hcy] = 1 mM.
**Fig. S3** The effect of pH values on the fluorescence intensity of PZ–Py (5 μM) in the absence or presence of ClO⁻ (50 μM). \( \lambda_{ex} = 400 \text{ nm; } \lambda_{em} = 562 \text{ nm.} \)

**Fig. S4** Fluorescent intensities of the probe PZ–Py (5 μM) upon addition of ClO⁻ (0–200 μM). \( \lambda_{ex} = 400 \text{ nm; } \lambda_{em} = 562 \text{ nm.} \)
Fig. S5 Time-dependent change of fluorescence intensity of PZ–Py (5 μM) upon addition of ClO⁻ (50 μM). \( \lambda_{ex} = 400 \text{ nm}; \lambda_{em} = 562 \text{ nm.} \)

![Graph showing fluorescence intensity over time.](image)

Fig. S6 The titration curve plotted with the fluorescence intensity of PZ–Py (5 μM) at 562 nm as a function of ClO⁻ concentration in range of 0–80 μM.

![Graph showing titration curve.](image)

### Table S1

<table>
<thead>
<tr>
<th>Parameter</th>
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Fig. S7 Absorption spectra of the probe PZ–Py (100 μM) upon additions of NaClO (0, 1, 2, 4, 6, 8, 10, 12, 14, 16, 20 equiv.) in PBS (pH 7.3, 10 mM, containing 1 % DMSO).

Fig. S8 HR-MS spectrum (positive mode) of product of PZ–Py reacted with ClO⁻
Fig. S9 $^{13}$C NMR spectrum of the product of PZ–Py reacted with ClO$^-$

Fig. S10 $^1$H NMR spectrum of the product of PZ–Py reacted with ClO$^-$
**Fig. S11** A possible product of the probe PZ–Py oxidized by ClO⁻

![Chemical structure](image)

**Fig. S12** Cytotoxicity of PZ–Py at various concentrations (2.5 μM, 5 μM, 10 μM, 15 μM and 25 μM) in living RAW 264.7 cells for 12 h.
**Fig. S13** (a–d) Confocal fluorescence images of HeLa cells. The cells were incubated with PZ–Py (5 μM) for 10 min, further incubated with NaClO (20 μM) for 15 min, and finally incubated with Mito Tracker Green (100 nM) for 20 min. (a) emission from the yellow channel, $\lambda_{ex}$=405 nm, $\lambda_{em}$: 531–590 nm; (b) emission from the green channel (mitochondrial staining, $\lambda_{ex}$=488 nm, $\lambda_{em}$: 500–530 nm); (c) merged image of a and b; (d) merged image of c and bright-field image. (e) Intensity profile of ROIs across HeLa cells. Red lines represent the intensity of the probe PZ–Py and blue lines represent the intensity of Mito Tracker Green. (f) Correlation plot of Mito Tracker Green and PZ–Py intensities.

**Fig. S14** Representative fluorescence images (pseudocolor) of a nude mouse (control group). The mouse was given a skin-pop injection of saline (100 μL) for 12 h, then a injection of saline (50 μL) for 30 min, finally a skin-pop injection of PZ–Py (50 μL×20 μM). Images were taken after incubation for 0, 5, 10, 15, 20, 30, 45 and 60 min, respectively. Images were taken using an excitation laser of 430 nm and an emission filter of DsRed channel.
ESI-MS spectrum of the compound 2

$^1$H NMR spectrum of compound 3
ESI-MS spectrum of the compound 3

$^1$H NMR spectrum of the probe PZ–Py
$^{13}$C NRM spectrum of the probe PZ–Py

HR-MS spectrum (positive mode) of the probe PZ–Py