Supporting Information for

Two-photon Fluorescent Probe for Revealing Drug-induced Hepatotoxicity via Mapping Fluctuation of Peroxynitrite

Yong Li†, Xilei Xie†, Xiu’e Yang, Mengmeng Li, Xiaoyun Jiao, Yuhui Sun, Xu Wang*, and Bo Tang*

College of Chemistry, Chemical Engineering and Materials Science, Collaborative Innovation Center of Functionalized Probes for Chemical Imaging in Universities of Shandong, Key Laboratory of Molecular and Nano Probes, Ministry of Education, Institute of Molecular and Nano Science, Shandong Normal University, Jinan 250014, P. R. China

E-mail: tangb@sdnu.edu.cn, wangxu@sdnu.edu.cn.

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1. Materials and instruments

Unless otherwise stated, all reagents for synthesis were purchased from commercial suppliers and were used without further purification. 3-Morpholino sydnonimine hydrochloride (SIN-1), which generates both superoxide anion and nitric oxide that spontaneously form peroxynitrite (ONOO⁻), was purchased from Sigma Chemical Company. Acetaminophen (APAP) was bought from 9 Ding Chemistry (Shanghai) Co., Ltd. Tolcapone was purchased from Bepharm (Shanghai) Ltd. N-acetyl cysteine (NAC) was purchased from Aladdin Industrial Corporation (Shanghai). Anti-
nitrotyrosine primary antibody was bought from Santa Cruz Biotechnology, Inc. Secondary antibody a Mo IgG (H+L) / TRITC was bought from Zhongshan Gold Bridge Biotechnology Co., Ltd. Sartorius ultrapure water (18.2 MΩ·cm) was used throughout the experiments. The probe was dissolved in dimethyl sulfoxide (DMSO) to produce 1.0 mM stock solution. Male Kunming mice (20 g) were purchased from School of Medicine at Shandong University. All animal experiments were in agreement with the guidelines of the Institutional Animal Care and Use Committee.

Fluorescence spectra were obtained with a FLS-980 fluorescence spectrometer (Edinburgh Instruments Ltd., England). Absorption spectra were recorded on a UV-1700 spectrophotometer (Shimadzu, Japan). pH measurements were performed with a pH-3c digital pH-meter (Shanghai Lei Ci Device Works, Shanghai, China). ¹H NMR and ¹³C NMR spectra were taken on a 400 MHz spectrometer (Bruker Co., Ltd., Germany), δ values are in ppm relative to TMS. HR-MS spectra were obtained on a maxis ultra-high resolution-TOF MS system (Bruker Co., Ltd., Germany). MTT assay was performed using a TRITURUS microplate reader. The HepG2 cells were maintained with incubator MCO-15AC (SANYO, Japan). The two-photon fluorescence images were taken using a LSM 880 confocal laser scanning microscopy (Zeiss Co., Ltd. Germany). Hematoxylin-eosin (H&E) staining images were obtained with Leica DMI3000B (Germany). Immunohistochemistry images were acquired using an LSM 880 confocal laser scanning microscopy (Zeiss Co., Ltd. Germany).
2. Synthesis

2.1 Synthesis of compound 1

Scheme S1. The synthesis of compound 1 and TP-KA.

To a solution of β-alanine tert-butyl ester hydrochloride (1.5 g, 10.0 mmol) in 5.0 mL DMF was added 4-amino-1,8-naphthalic anhydride (213 mg, 1.0 mmol). The mixture was heated at 135 °C under the flow of Ar gas for 1 h and then concentrated under reduced pressure. The residue was purified by column chromatography on silica gel with ethyl acetate/petroleum ether (1:3, v/v) to afford 1 as a flavor-green solid (280 mg, 82.4% yield). $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 8.62 (d, $J = 8$ Hz, 1H), 8.42 (d, $J = 8$ Hz, 1H), 8.19 (d, $J = 8$ Hz, 1H), 7.65 (t, $J = 8$ Hz, 1H), 7.47 (s, 2H), 6.84 (d, $J = 8$ Hz, 1H), 4.23 (t, $J = 8$ Hz, 2H), 2.53 (t, $J = 8$ Hz, 2H), 1.33 (s, 9H). $^{13}$C NMR (100 MHz, DMSO-$d_6$) $\delta$ 170.67, 164.14, 163.20, 153.23, 134.42, 131.45, 130.19, 129.83, 124.42, 122.18, 119.82, 108.64, 107.94, 80.42, 35.84, 34.15, 28.04. HRMS (ESI): calculated for C$_{19}$H$_{20}$N$_2$O$_4$ [M+Na]$^+$ = 363.1315, Found 363.1270.

2.2 Synthesis of TP-KA

A mixture of 2-(4-nitrophenyl)-2-oxoacetic acid (195 mg, 1.0 mmol), oxalyl chloride (265 μL, 3.0 mmol) and DMF (2 drops) in dichloromethane (3.0 mL) was refluxed for 1 h and then evaporated. To this crude product, 5 mL dichloromethane was added, and then compound 1 (34 mg, 0.1 mmol) and triethylamine (12 μL, 0.4 mmol) were added. The reaction mixture was stirred at room temperature for 30 min.
After that, the mixture was concentrated under vacuum, and the residue was purified by silica chromatography eluted with ethyl acetate/petroleum ether (1:8, v/v) to afford **TP-KA** as an orange-yellow solid (40.0 mg, 77.3% yield). \(^1\)H NMR (400 MHz, DMSO-\(d_6\)) \(\delta\) 11.53 (s, 1H), 8.64 (d, \(J = 8\) Hz, 1H), 8.57 (t, \(J = 8\) Hz, 2H), 8.43 (d, \(J = 8\) Hz, 2H), 8.38 (d, \(J = 8\) Hz, 2H), 8.30 (d, \(J = 8\) Hz, 1H), 7.93 (t, \(J = 8\) Hz, 1H), 4.28 (t, \(J = 8\) Hz, 2H), 2.60 (t, \(J = 8\) Hz, 2H), 1.34 (s, 9H). \(^{13}\)C NMR (100 MHz, DMSO-\(d_6\)) \(\delta\) 187.21, 170.56, 164.92, 163.66, 151.10, 137.35, 132.02, 131.60, 131.54, 131.12, 129.89, 128.64, 127.40, 125.54, 124.54, 124.40, 122.05, 119.75, 80.55, 36.22, 33.86, 28.04. HRMS (ESI): calculated for C\(_{27}\)H\(_{23}\)N\(_3\)O\(_8\) [M−H]\(^−\) = 516.1401, Found 516.1462.

3. Preparation of various ROS and RNS solutions

Hydrogen peroxide (H\(_2\)O\(_2\)), hypochlorite (ClO\(^−\)) and tert-butyl hydroperoxide (TBHP) were delivered from commercial aqueous solutions respectively. Superoxide solution (O\(_2\)•\(^−\)) was prepared by adding KO\(_2\) into dry dimethylsulfoxide and stirring vigorously for 10 min. Singlet oxygen (\(^1\)O\(_2\)) was generated in situ by addition of the H\(_2\)O\(_2\) stock solution into a solution containing 10 eq of HClO. Hydroxyl radicals (•OH) was generated by Fenton reaction. Nitric oxide (NO) was used from a stock solution prepared by SNP (sodium nitroferricyanide (III) dihydrate). Peroxynitrite solution (ONOO\(^−\)) was synthesized as reported literature. Briefly, a mixture of sodium nitrite (0.6 M) and hydrogen peroxide (0.7 M) was acidified with hydrochloric acid (0.6 M), and sodium hydroxide (1.5 M) was added within 1–2 s to make the solution alkaline. The peroxynitrite concentration was estimated by using an extinction coefficient of 1670 M\(^−1\)cm\(^−1\) at 302 nm. \(C_{\text{ONOO}}\(^−\) = \text{Abs}_{302\ \text{nm}} /1.67\) (mM).

4. Measurement of two-photon absorption cross-section (\(\delta\))

Utilizing fluorescein as the reference molecules, whose two-photon property has been well characterized in the literature. The two-photon absorption cross-section (\(\delta\))
of TP-KA and compound 1 were determined. The two-photon absorption cross-section was calculated by using equation (1):

\[
\delta_s = \frac{F_r \Phi_r n_r C_r}{F_s \Phi_s n_s C_s}
\]

The subscripts \( s \) and \( r \) refer to the sample and the reference molecules, respectively. \( \delta \) is the two-photon absorption cross sectional value, the compounds concentration in solution was denoted as \( C \), \( n \) is there refractive index of the solution, \( F \) is two-photon excited fluorescence integral intensity and \( \Phi \) is the fluorescence quantum yield. Here, the fluorescence quantum yield of TP-KA and compound 1 were measured as 0.082 and 0.714, respectively.

5. MTT assays

The MTT assay was conducted to evaluate the cytotoxicity of TP-KA. Hepatic cells were replanted in the 96-well micro plates to a total volume of 200 µL well\(^{-1}\). The plates were maintained at 37°C, 5% CO\(_2\)/95% air incubator for 24 hours. Then, the cells were incubated with different concentrations TP-KA (0, 1.0, 10.0, 30.0, 50.0 and 100.0 µM) for another 12 hours. Subsequently, the culture medium was removed and MTT solution (5.0 mg ml\(^{-1}\)) was added to each well. After 4 hours, the remaining MTT solution was removed and 150 µL DMSO was added to dissolve the formazan crystals. The absorbance of solution was measured at 490 nm with 5 min gentle agitation using a TRITURUS microplate reader.

6. Two-photon fluorescence confocal imaging in cells

HepG2 cells were seeded into 18 mm glass-bottom dishes with high-glucose DMEM medium containing 10% fetal bovine serum, 1% penicillin, and 1% streptomycin. Cultures were maintained in incubator (37°C, 5% CO\(_2\)/95% air) for 24 hours. The cells were excited at 800 nm with a two-photon laser and the emission was collected between 500–600 nm.
For exogenous ONOO$^-$ imaging experiments, after washed with PBS for three times, HepG2 cells were incubated with 1.0 mM SIN-1 for 30 min followed by treated with 1.0 μM TP-KA for 20 min. As to ONOO$^-$ elimination assays, the cells were treated with 100 μM uric acid for 3 h after incubated with 1.0 mM SIN-1 for 30 min, and then co-incubated with 1.0 μM TP-KA for 20 min. Cell imaging was carried out after washing the cells with PBS for three times.

For the drug-induced cell damage imaging, after washed with PBS for three times, HepG2 cells were treated with APAP (125 μM and 250 μM, respectively) or tolcapone (125 μM and 250 μM, respectively) for 1 h, and then incubated with 1.0 μM TP-KA for 20 min before imaging. For the remediation investigation, HepG2 cells were pretreated with 1 mM NAC for 1 h before drug administration.

7. Two-photon fluorescence confocal imaging in tissues

Kunming mice (~20 g) were fasted for 12 h to avoid the possible food fluorescence interference. The mice were injected intraperitoneally with 300 μL PBS buffer solution containing APAP or tolcapone (0, 100 mg/kg and 300 mg/kg, respectively). After 30 min, 50 μM TP-KA in 100 μL PBS buffer were subsequently injected through the tail-vein. Thirty minutes later, all the mice were dissected to isolate the livers, which were cut into slices for confocal imaging. The tissue imaging was excited at 800 nm with a two-photon laser and the emission was collected between 500–600 nm. For the remediation investigation, 300 μL PBS buffer containing NAC (300 mg/kg) was injected intraperitoneally 1 h before drug administration.

8. Histology and Immunohistochemistry staining assays

Kunming mice (~20 g) were fasted for 12 h to avoid the possible fluorescence interference. The mice were injected intraperitoneally with 300 μL PBS buffer solution containing APAP or tolcapone (0, 100 mg/kg, and 300 mg/kg, respectively). After 3 h, the mice were dissected to isolate the livers, which were then fixed with 4% PFA for 12 h. Then the samples were dehydrated with ethanol and embedded in
paraffin before 6 µm sectioning. For the remediation investigation, 300 µL PBS buffer containing NAC (300 mg/kg) was injected intraperitoneally 1 h before drug administration.

H&E staining samples were prepared from the mice stained by hematoxylin and eosin under standard protocols. As to immunohistochemistry staining assays, the sections were washed with PBS buffers for three times and blocked with normal goat serum for 1 h. And then, an anti-nitrotyrosine primary antibody was utilized to label nitrotyrosine overnight, followed by colored with Rhodamine (TRITC)-labeled secondary antibody for 1 h. After that, the sections were imaged by excited at 543 nm with an LSM 880 confocal laser scanning microscopy and the emission was collected between 580–640 nm.

9. Two-photon fluorescence confocal imaging in vivo

Kunming mice (~20 g) were fasted for 12 h to avoid the possible fluorescence interference. 300 µL PBS buffer solution containing 300 mg/kg tolcapone was injected intraperitoneally and 50 µM TP-KA in 100 µL PBS buffer was injected through the tail-vein. For the remediation investigation, 300 µL PBS buffer containing NAC (300 mg/kg) was injected intraperitoneally 1 h before drug administration. As to ONOO− clearance assay, the mice were injected intraperitoneally with 300 µL PBS buffer solution containing uric acid (300 mg/kg) 1 h before drug administration. Then, all the mice were anaesthetized and performed surgical procedure to expose the liver. The liver was imaged every 5 min with excitation at 800 nm and the emission was collected between 500–600 nm.
Figure S1. The UV-vis (a) and fluorescent emission (b and c) spectra of TP-KA and compound 1 (both concentration of TP-KA and 1 were 45.0 μM) in PBS buffer (50 mM, pH 7.4) with 1% DMSO. Figure (b) and Figure (c) were obtained by excited at 375 nm and 430 nm, respectively. Slit width: 2.5 nm/2.5 nm.

Figure S2. Fluorescence intensity changes of TP-KA (1.0 μM) upon treated with H$_2$O$_2$ (200 μM) and ONOO$^-$ (10 μM) in PBS buffer (50 mM, pH 7.4) with 1% DMSO. \( \lambda_{ex}/\lambda_{em} = 430/560 \text{ nm} \).

Figure S3. Time-dependent fluorescence changes of TP-KA (1.0 μM) toward
ONOO\(^-\) (10 \(\mu\)M) in PBS buffer (50 mM, pH 7.4) at 37 °C with 1% DMSO. \(\lambda_{ex}/\lambda_{em} = 430/560\) nm.

**Figure S4.** Fluorescence intensity change of TP-KA (1 \(\mu\)M) as the addition ONOO\(^-\) (10 \(\mu\)M) under different pH values (5.0, 6.0, 7.0, 7.4, 8.0, 9.0) by using universal buffer solution (0.1 mM citric acid, 0.1 M KH\(_2\)PO\(_4\), 0.1 M Na\(_2\)B\(_4\)O\(_7\), 0.1 M Tris, 0.1 M KCl) with 1% DMSO. \(\lambda_{ex}/\lambda_{em} = 430/560\) nm.

![Figure S4](image)

**Figure S5.** HRMS spectral analysis of reaction between TP-KA and ONOO\(^-\).

![Figure S5](image)
Scheme S2. The proposed reaction mechanism of TP-KA with ONOO⁻.

Figure S6. MTT assay of HepG2 cells with different concentrations of TP-KA.
**Figure S7.** Representative nitrotyrosine staining images of the liver of mice treated with various conditions. (a) and (e) PBS buffer only as control group; (b) and (c) APAP (100 mg/kg and 300 mg/kg, respectively); (d) NAC (300 mg/kg) pretreated and then APAP (300 mg/kg); (f) and (g) tolcapone (100 mg/kg and 300 mg/kg, respectively); (h) NAC (300 mg/kg) and then tolcapone (300 mg/kg). Excitation wavelength: 543 nm, and emission: 580–640 nm. Scar bar = 50 µM.
NMR spectra of compound 1 and TP-KA

Figure S8. $^1$H and $^{13}$C NMR spectra of compound 1 (DMSO-$d_6$).
Figure S9. $^1$H and $^{13}$C NMR spectra of TP-KA (DMSO-$d_6$).