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Research Paper

Mitochondria-targeted two-photon fluorescent probe for the detection of biothiols in living cells

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ABSTRACT

Mitochondrial thiols play a key role in maintaining the redox environment. In this work, three mitochondria-targeted two-photon probes (**1**–**3**) have been explored to detect biothiols in vitro and in vivo with a large turn-on fluorescence signal. These fluorescence probes display sensitive and selective response to thiols in the water environment, feature good linearity ranges with low detection limit. The negligible cytotoxicity, excellent photophysical properties and high selectivity of these probes will find their useful applications in biomedical research.

1. Introduction

Intracellular thiols such as glutathione (GSH), homocysteine (Hcy) and cysteine (Cys) play a crucial role in numerous biological processes [1–3]. Mitochondria, an essential organelle within eukaryotic cell, generate much of the cellular energy, regulate the cellular redox state, emerge as promising pharmacological target in clinical applications for the detection [4], Moreover, mitochondria produce most of the cellular reactive oxygen species (ROS) [5], buffer cellular Ca^{2+} [6], and initiate cellular apoptosis [7,8]. As a primary site of oxygen consumption and the major source of ROS, mitochondrial thiols play an important role in maintaining the redox equilibrium to avoid or repair oxidative damage leading to dysfunction and cell death [8–10]. Hence, to understand the roles of mitochondria thiols in biology, real-time detailed monitoring of these thiols is urgently necessary, which has significant effect on mitochondrial associated diseases [11,12]. For this purpose, a variety of fluorescence probes have been developed [1,9,13–25].

Considering that the mitochondrial membrane spans across a negative potential, most mitochondria-targeted agents have a positively charged moiety, such as proteins, peptides, peptide mimetics and lipophilic cationic compounds [26]. Among the mitochondria-targeted lipophilic cationic compounds, the triphenylphosphonium (TPP) cations compounds have been widely used [27–32]. However, the peptidic compounds are difficult to synthesize, and the TPP is difficult for further chemical modifications [33,34]. In addition, most of these probes have been evaluated as one-photon microscopy (OPM). The OPM needs shorter excitation wavelength, which limits its application in live deep tissue and cell imaging due to shallow penetration depth of excitation light [35]. Comparing with OPM, the two-photon fluorescence microscopy (TPM) exciting the fluorophores with NIR laser pulses, can provide improved three-dimensional spatial localization and increased imaging depth with focused excitation and reduced photo bleaching [36–41]. To the best of our knowledge, although fluorescent probes for biothiols have been widely developed, two-photon probe for mitochondrial thiols is still rare [13]. In addition, the thiols assisted removal of 2,4-dinitrobenzenesulfonyl (DNBS) from the hydroxyl group moiety of a fluorophore has been found to have excellent selectivity towards thiols detection with turn–on fluorescence signaling [42–45]. Therefore, it is feasible to develop selective two-photon probe for mitochondrial thiols in living cells.

In this work, a series of thiols responsive probes **1**–**3** were designed, synthesized and characterized. The optical properties, in vitro cytotoxicity, mitochondrial imaging, selective response and mechanism were investigated. Cellular imaging experiments clearly indicated that these probes can selectively respond to mitochondrial thiols in living cells (Fig. 1).

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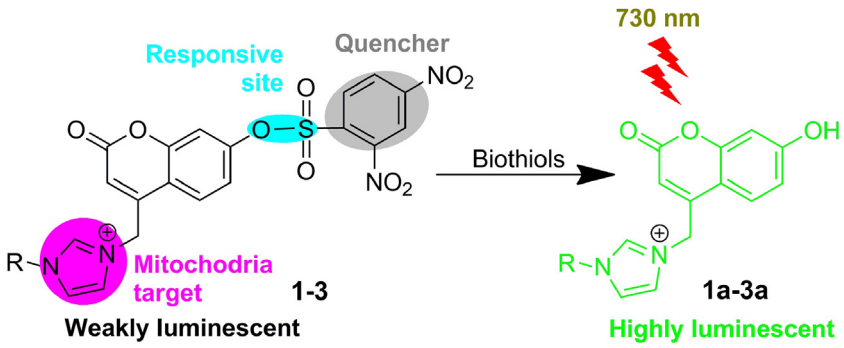

Fig. 1. Schematic representation of the design and response mechanism of these compounds toward biothiols.

2. Experimental section

2.1. General materials and methods

All starting materials were used as received from commercial sources unless otherwise indicated. Solvents were purified by standard procedures. The 4-(chloromethyl)-7-hydroxycoumarin (**CMHC**) was synthesized according to the literature procedures [46]. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma Aldrich), DMSO (Sigma Aldrich), Cisplatin (Sigma Aldrich), NEM (N-methylmaleimide, Sigma Aldrich), MTR (Mitotracker Red, Life Technologies, USA) were used as received. The tested compounds were dissolved in DMSO just before the experiments, and the concentration of DMSO was 1% (v/v). ^{1}H NMR and ^{13}H NMR spectra were recorded on a Mercury Plus 300 spectrometer. Shifts are referenced relative to the internal solvent signals. Microanalysis was carried out using an Elemental Vario EL CHNS analyzer. Fluorescence spectra were recorded on a Shimadzu RF5301 spectrofluorophotometer. UV–vis spectra were recorded on a Varian Cary 300 spectrophotometer. ESI-MS were recorded on a Thermo Finnigan LCQ DECA XP spectrometer. The quoted *m*/*z* values represent the major peaks in the isotopic distribution. Fluorescence microscopy of cells was performed in Carl Zeiss LSM 710. For MTT assays, the absorbance was quantified using Infinite M200 microplate reader.

2.2. Synthesis and characterization

TPP-HC: Triphenylphosphine (525 mg, 2 mmol) in THF was added dropwise to a solution of **CMHC** (211 mg, 1 mmol) in THF. The reaction mixture was refluxed for 2 days to give a white precipitate. The suspension was filtered, washed with THF, and then dried under vacuum (320 mg, yield 67.7%). ^{1}H NMR (300 MHz, DMSO) δ 10.83 (s, 1H), 7.93–7.79 (m, 9H), 7.72 (dt, J = 11.0, 5.6 Hz, 6H), 7.24 (d, J = 8.9 Hz, 1H), 6.69 (d, J = 0.9 Hz, 1H), 6.37 (d, J = 8.8 Hz, 1H), 5.93 (d, J = 3.9 Hz, 1H), 5.39 (d, J = 16.9 Hz, 2H). ^{13}C NMR (75 MHz, DMSO) δ 162.25, 159.57, 155.29, 144.91, 134.67, 130.69, 127.44, 117.98, 117.12, 114.88, 112.85, 110.87, 102.83, 25.07. ESI–MS: calcd. for [M-Cl]$^+$ *m*/*z* 437.5. Found: [M-Cl]$^+$ *m*/*z* 437.2. Elemental analyses calcd (%) for $C_{28}H_{22}ClO_3P$: C, 71.11; H, 4.69; found C, 71.30; H, 4.61.

Synthetic procedure of the compounds **1a**–**3a**: 1-alkyl-1H-imidazole (20 mmol) in THF was added dropwise to a solution of 4-(chloromethyl)-7-hydroxycoumarin (1.053 g, 5 mmol) in THF. The reaction mixture was refluxed for 2 days to give a white precipitate. The suspension was filtered, washed with THF, and then dried under vacuum.

1a (1.352 g, yield 92.4%). ^{1}H NMR (300 MHz, DMSO) δ 10.90 (s, 1H), 9.25 (s, 1H), 7.83 (s, 1H), 7.78 (s, 1H), 7.66 (d, J = 8.4 Hz, 1H), 6.88 (d, J = 8.9 Hz, 1H), 6.82 (s, 1H), 5.79 (s, 1H), 5.75 (s, 2H), 3.89 (s, 3H). ^{13}C NMR (75 MHz, DMSO) δ 163.87, 161.74, 156.85, 151.41, 139.60, 127.55, 126.06, 124.83, 115.23, 110.99, 110.80, 104.49, 50.14, 37.96. ESI–MS: calcd. for [M-Cl]$^+$ *m*/*z* 257.3. Found: [M-Cl]$^+$ *m*/*z* 257.0. Elemental analyses calcd (%) for $C_{14}H_{13}ClN_2O_3{\cdot}0.5H_2O$: C, 55.82; H, 4.67; N, 9.18; found C, 55.73; H, 4.68; N, 9.28.

2a (1.283 g, yield 83.1%). ^{1}H NMR (300 MHz, DMSO) δ 11.04 (s, 1H), 9.42 (s, 1H), 7.92 (s, 1H), 7.88 (d, J = 1.5 Hz, 1H), 7.67 (d, J = 8.7 Hz, 1H), 6.90 (dd, J = 8.7, 2.3 Hz, 1H), 6.84 (d, J = 2.3 Hz, 1H), 5.80 (s, 1H), 5.76 (s, 2H), 4.23 (q, J = 7.3 Hz, 2H), 1.44 (t, J = 7.3 Hz, 3H). ^{13}C NMR (75 MHz, DMSO) δ 163.90, 161.74, 156.86, 151.25, 138.80, 127.54, 125.01, 124.54, 115.24, 111.29, 110.80, 104.51, 50.18, 46.41, 16.62. ESI–MS: calcd. for [M-Cl]$^+$ *m*/*z* 271.3. Found: [M-Cl]$^+$ *m*/*z* 271.1. Elemental analyses calcd (%) for $C_{15}H_{15}ClN_2O_3{\cdot}0.2H_2O$: C, 58.29; H, 4.99; N, 8.97; found C, 58.05; H, 5.00; N, 9.03.

3a (1.633 g, yield 96.9%). ^{1}H NMR (300 MHz, DMSO) δ 11.02 (s, 1H), 9.42 (s, 1H), 7.90 (s, 1H), 7.89 (d, J = 1.6 Hz, 1H), 7.65 (d, J = 8.7 Hz, 1H), 6.89 (dd, J = 8.7, 2.3 Hz, 1H), 6.84 (d, J = 2.3 Hz, 1H), 5.80 (s, 1H), 5.76 (s, 2H), 4.20 (t, J = 7.2 Hz, 2H), 1.80 (dt, J = 14.7, 7.4 Hz, 2H), 1.25 (td, J = 14.7, 7.3 Hz, 2H), 0.90 (t, J = 7.3 Hz, 3H). ^{13}C NMR (75 MHz, DMSO) δ 163.98, 161.74, 156.85, 151.19, 139.09, 127.49, 125.06, 124.82, 115.25, 111.33, 110.73, 104.51, 50.74, 50.21, 32.94, 20.63, 15.08. ESI-MS: calcd. for [M-Cl]$^+$ *m*/*z* 299.3. Found: [M-Cl]$^+$ *m*/*z* 299.1. Elemental analyses calcd (%) for $C_{17}H_{19}ClN_2O_3$: C, 60.59; H, 5.78; N, 8.25; found C, 60.99; H, 5.72; N, 8.37.

Synthetic procedure of the compounds **1**–**3**: A solution of 2,4-dinitro-benzenesulfonylchlorid (480 mg, 1.8 mmol) in THF (10 mL) was added dropwise to a solution of **1a**–**3a** (1.5 mmol) and Na_2CO_3 (80 mg, 0.75 mmol) in water (3 mL). The reaction mixture was refluxed for 3 days to give a white precipitate. The suspension was filtered, washed with THF, and then dried under vacuum.

1 (305 mg, yield 38.9%). ^{1}H NMR (300 MHz, DMSO) δ 9.15 (s, 1H), 9.13 (d, J = 2.3 Hz, 1H), 8.63 (dd, J = 8.7, 2.2 Hz, 1H), 8.53 (d, J = 2.3 Hz, 1H), 8.38 (dd, J = 8.5, 2.2 Hz, 1H), 8.33 (d, J = 8.7 Hz, 1H), 8.06 (d, J = 8.6 Hz, 1H), 7.91 (d, J = 8.8 Hz, 1H), 7.46 (d, J = 2.4 Hz, 1H), 6.13 (s, 1H), 5.78 (s, 2H), 3.88 (s, 3H).^{13}C NMR (75 MHz, DMSO)δ 158.64, 153.62, 151.59, 150.28, 148.07, 147.35, 144.72, 137.75, 133.47, 130.71, 127.66, 126.58 125.55, 122.84, 121.25, 118.35, 118.20, 110.88, 48.18, 36.10.ESI-MS: calcd. for [M-Cl]$^+$ *m*/*z* 487.42. Found: [M-Cl]$^+$ *m*/*z* 486.60. Elemental analyses calcd (%) for $C_{20}H_{15}ClN_4O_9S{\cdot}3H_2O$: C, 41.64; H, 3.67; N, 9.71; found C, 41.46; H, 3.82; N, 9.53.

2 (363 mg, yield 45.1%). ^{1}H NMR (300 MHz, DMSO) δ 9.23 (s, 1H), 9.12 (d, J = 2.3 Hz, 1H), 8.63 (dd, J = 8.7, 2.3 Hz, 1H), 8.53 (d, J = 2.3 Hz, 1H), 8.38 (dd, J = 8.6, 2.3 Hz, 1H), 8.33 (d, J = 8.7 Hz, 1H), 8.06 (d, J = 8.6 Hz, 1H), 7.91 (d, J = 4.8 Hz, 1H), 7.46 (d, J = 2.4 Hz, 1H), 6.16 (s, 1H), 5.76 (s, 2H), 4.23 (q, J = 7.3 Hz, 2H), 1.45 (t, J = 7.3 Hz, 3H). ^{13}C NMR (75 MHz, DMSO)δ 158.64, 153.64, 151.59, 150.28, 148.16, 147.36, 144.70, 136.90, 133.49, 130.71, 127.67, 126.57, 125.56, 122.79, 121.24, 118.46, 118.24, 110.89, 48.21, 44.59, 14.66.ESI-MS: calcd. for [M-Cl]$^+$ *m*/*z* 501.45. Found: [M-Cl]$^+$ *m*/*z* 501.10. Elemental analyses calcd (%) for $C_{21}H_{17}ClN_4O_9S{\cdot}3H_2O$: C, 42.68; H, 3.92; N, 9.48; found C, 42.43; H, 4.15; N, 9.22.