Mitochondria-targeting near-infrared ratiometric fluorescent probe for selective imaging of cysteine in orthotopic lung cancer mice

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\textbf{ABSTRACT}

Cysteine (Cys) plays significant roles in many physiological processes, although its normal concentration is maintained at the micromole level. Abnormally high levels of intracellular Cys can lead to many diseases including cancer. Recent years, many effective fluorescent probes have been developed for the selective detection of Cys against other biological thiols. Herein, we synthesized a ratiometric near-infrared (NIR) fluorescent probe Cy-OAcr for selective imaging of intracellular Cys. Cy-OAcr has a lipophilic iminium cation unit as the mitochondrial guider and an acrylate group as the Cys recognition unit as well as a fluorescence modulator for rearranging the conjugated π-electron system of cyanine fluorophore. Upon detection of Cys, there occurs a significant absorption and fluorescence spectral shift, which are desirable for ratiometric detection. This probe has high sensitivity and selectivity for Cys detection over glutathione (GSH), homocysteine (Hcy), and other biomolecules with a low limit of detection at 0.09 μM. Probe Cy-OAcr is capable to detect and image Cys in three living cancer cell lines and their corresponding tumor-bearing mice models. More importantly, we successfully apply this fluorescent probe to evaluate the level of Cys in orthotopic lung cancer model. Imaging analyses reveal that the probe can discriminate tumor lesions from normal tissues, indicating its significant potential applications for clinical auxiliary diagnosis of cancer.

\section{1. Introduction}

As an important biological thiol, cysteine (Cys) plays vital roles in the biological organisms. Once incorporated into cells, most of Cys is immediately involved in the synthesis of glutathione (GSH). However, Cys holds an independent redox pool to regulate mitochondrial dynamic antioxidant defenses compared to the antioxidant system of GSH [1]. The severe level changes of intracellular Cys will result in many diseases. The abnormally high concentrations of Cys has relationship with cardiovascular disease, neurotoxicity, Alzheimer's disease, and Parkinson's disease, while the deficiency will cause edema, alopecia, and liver damage, although the actual cause of those diseases is still debatable [2]. Especially in cancer cells, the change of Cys concentration is much higher than that of GSH [3]. That is, cancer tissue has significantly higher levels of Cys than adjacent normal tissue [4]. The determine of the levels of intracellular Cys can potentially discriminate tumor lesions from adjacent more normal tissue. However, the detection of Cys is always challenged by GSH and homocysteine (Hcy) because of the similar chemical structure and properties (Supporting information, Fig. S2) [5,6]. Moreover, the levels of Cys, Hcy and GSH in cells are very different. GSH is the most abundant non-protein thiol in cell, the concentration is at about micromole level [1,7]. Therefore, it is full of challenges to develop the selective and sensitive detection method of Cys against other biothiols such as Hcy and GSH.

Previous efforts on Cys detection, including high performance liquid chromatography (HPLC) [8], mass spectrometry (MS) [9], HPLC-MS [10], immunoassay [11], and capillary electrophoresis separations
[12,13] methods, often involve time-consuming and complicated separate steps [14]. Fluorescent probes combined with imaging techniques has now become indispensable chemical tools for the detection of reactive biological small molecules in living biosystems due to the high sensitivity and unrivaled spatiotemporal resolution [15,16]. Current fluorescent probes are mainly designed for specific identification of Cys, Hcy and GSH in cells [17–31]. The fluorescence response mechanisms involve Förster resonance energy transfer mechanism [32], photo-induced electron transfer mechanism [33–35], intramolecular charge transfer mechanism [36,37], as well as aggregation-induced emission mechanism [38,39], and so on. The type of reaction detection mechanisms are commonly adopted Michael addition [40], cyclization reaction [41], cleavage reaction [42], conjugated addition-cyclization reaction [43], cascade reaction [44] and substitution-rearrangement reaction [45].

Although many fluorescent probes have made a significant contribution to the detection of Cys, there still exist many issues to be solved. The fluorescent probes whose emission locate in the ultraviolet or visible region are limit by penetration and photobleaching for bioimaging in cells, particularly in vivo [46,47]. The reported probes which based the BODIPY and coumarin fluorophores have failed to target mitochondria for Cys detection [17,28,32]. More importantly, most of these fluorescent probes often achieve Cys detection only at the cell level [16,17,30,39]. In order to further reveal the roles of Cys in pathogenesis and physiology, it is necessary to design and synthesize an effective fluorescent probe for in real-time imaging of the accurate distribution of Cys in cells and in animal models. Moreover, the NIR fluorescence can maximize tissue penetration while minimizing the absorbance of heme in hemoglobin and myoglobin, water, and lipids, therefore, the NIR fluorescent probes are more desirable for in real-time imaging the distribution of Cys in cells and in vivo [50–52]. In order to be satisfied precise fluorometric analyses under biological conditions, the ratiometric probes which employ the ratio of the emission intensity at two different wavelengths are more preferred, because interferences from uneven loading or the inhomogeneous distribution of fluorescent probes in cells can be reduced [53,54]. After scanning the above mentioned issues for Cys detection, Yoon et al reported a near-infrared ratiometric fluorescence probe CyAC for the detection of Cys in cells and in vivo [55].

Herein, in view of the excellent performance of CyAC, we further synthesized a ratiometric NIR fluorescent probe Cy-OAcr for the detection of mitochondrial Cys over GSH and Hcy in cells and in orthotopic lung cancer mice. The probe Cy-OAcr was able to quickly react with Cys and produce a shift in fluorescence emission profile, meanwhile avoiding the interference from other small endogenous reactive small molecules. The probe could be used to discriminate Cys levels in different types of cancer cell lines, and the minimum cation of this fluorophore platform could facilitate the probe accumulating in mitochondria. To the best of our knowledge, we first applied a Cys fluorescent probe for imaging of tumor lesions in orthotopic lung cancer models. We envisioned that the probe Cy-OAcr could contribute to further understanding the roles of Cys in physiological and pathological condition. And Cys might be a potential biomarker for the clinical diagnosis of tumor.

2. Experimental section

2.1. Synthesis of the probe Cy-OAcr

2.1.1. Synthesis of compound 2 (Keto-Cy)

To a solution of compound 1 (0.512 g, 1.0 mmol) and sodium acetate (0.164 g, 2 mmol) dissolved in anhydrous DMF (10 ml) under argon protection, the reaction mixture was reacted at 50 °C for 12 h [56,57]. The crude product was extracted and washed by water : dichloromethane = 1 : 1 (v/v) three times. The solvent was removed under reduced pressure. The resulting solid was purified by flash chromatography using dichloromethane : ethyl acetate = 10 : 1 (v/v) as eluent to afford red solid (0.43 g, yield: 87%). 1H NMR (DMSO-D6, 500 MHz) δ (ppm): 7.96–7.93 (d, 2 H), 7.34–7.33 (d, 2 H), 7.22–7.19 (m, 2 H), 6.92–6.89 (m, 4 H), 5.50–5.47 (d, 2 H), 3.83–3.79 (q, 4 H), 2.58–2.55 (t, 4 H), 1.76–1.74 (m, 2 H), 1.57 (s, 12 H), 1.18–1.13 (m, 6 H). 13C NMR (DMSO-D6, 125 MHz) δ (ppm): 188.26, 164.17, 145.11, 142.53, 133.23, 129.36, 123.81, 119.59, 109.73, 98.84, 50.08, 39.74, 29.06, 25.01, 20.05, 9.94. HRMS (ESI+): m/z C30H40N2O calculated 492.3141, found [M + H]+ 493.3142.

2.1.2. Synthesis of compound 3 (Cy-OAcr)

To a solution of acryloyl chloride (0.905 g, 10 mmol) in anhydrous dichloromethane (10 ml) under argon protection, compound 2 (0.493 g, 1 mmol) was drippedwise added [56]. The reaction mixture was lasted at 0 ºC for 1 h, and then heated to 25 ºC for 12 h. The crude product was washed by water three times. The solvent was removed under reduced pressure. The resulting solid was purified by flash chromatography using ethyl acetate : methanol = 1 : 1 (v/v) as eluent to afford green solid (0.498 g, yield: 91%). 1H NMR (DMSO-D6, 500 MHz) δ (ppm): 7.65–7.61 (m, 3 H), 7.45–7.44 (m, 2 H), 7.29–7.27 (m, 2 H), 6.88–6.72 (m, 2 H), 6.49–6.47 (m, 1 H), 6.28–6.25 (m, 3 H), 5.64–5.61 (m, 1 H), 5.90–5.88 (m, 1 H), 4.27–4.22 (m, 4 H), 2.76–2.71 (m, 4 H), 1.55 (s, 12 H), 1.33–1.30 (m, 8 H). 13C NMR (DMSO-D6, 125 MHz) δ (ppm): 121.90, 171.61, 167.35, 163.79, 158.73, 142.12, 141.54, 139.78, 136.51, 131.91, 130.05, 129.10, 127.11, 125.47, 123.02, 121.36, 111.74, 100.96, 49.25, 37.67, 27.83, 24.19, 20.97, 12.65. HRMS (ESI+): m/z C32H38N2O2 calculated 547.3319, found [M] 547.3320.

2.2. Cell culture

The A549 cell line, H1650 cell line and PC9 cell line were purchased from Chinese Academy of Sciences stem cell bank/stem cell technology platform. A549 cell line were incubated in F-12 K (Invitrogen, 21127-022) supplemented with 10% fetal bovine serum (FBS, Gibco), PC9 cell line were incubated in RPMI (gibco, 11875-093) supplemented with 10% FBS, H1650 cell line were incubated in DMEM (HyClone, SH30022.01) supplemented with 10% FBS. The cells grew in the incubator (37 °C, 5% CO2).

2.3. Construction of tumor-bearing mice model

BALB/c nude mice, female, 4 weeks old, were provided by Changzhou Cavens Lab Animal Co. Ltd. A549 cell line, H1650 cell line and PC9 cell line digested into suspension with concentration of 1×10⁷ /ml in serum-free medium for cell xenografts. Until the diameter of tumor reached about 6 mm can be experiment.

2.4. Construction of orthotopic lung cancer mice model

SPF grade FVB male mice purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. 6 weeks old, body weight 20 ± 2 g. Mice received i.p. injections of 8% urethane (dissolved in saline) at a dose of 0.01 ml g⁻¹ twice a week for 5 weeks [58]. Then it would take three months for the development of orthotopic lung cancer lesions. The weights of mice were monitored once a week when the model was started, and the growth status of mice was observed every day.

2.5. Cell counting Kit-8 (CCK-8) experiment

The growth adherent cells were digested into cell suspensions. The number of cells in the prepared cell suspension was counted by cell counting plates. Then, the cells were inoculated into 96 holes and the cell suspension 100 μl was added per hole. 96-well cell culture plate were cultured in a cell incubator for 24 h. Different concentrations (0–100 μM) of Cy-OAcr (10 μl) were added into the culture plate. The
culture plate was incubated in incubator for 24 h. Add 10 µl CCK-8 of solution to each hole (notice not to generate bubbles in the hole). The 96-well cell culture plate was incubated in the incubator for 30 min and then determined the absorbance value by microplate reader at 450 nm.

2.8. In vivo mice imaging

In vivo imaging of mice performed on Perkinelmer IVIS Lumina XRMS Series III In Vivo Imaging System. Fluorescence imaging detection gain remains constant throughout the experimental process.

2.6. Confocal laser scanning microscope imaging experiment

The cells which grew well were digested into cell suspension and inoculated into the Petridishes (Φ = 20 mm). The cells were incubated in the incubator for 24–48 h and then performed the confocal imaging with an objective lens (× 60). Fluorescence excitation and collection filters were described in the paper. Fluorescence imaging detection gain remains constant throughout the experimental process.

2.7. Flow cytometry analysis

Take logarithmic growth phase cells digested into cell suspension and inoculated into 6-well plates (define: 2.0 × 10^5 cells/well). The 6-well plates incubated for 24 h. Then treated with Cy-OAcrr as described in the paper. After the treatment was complete, the cells are washed with Phosphate Buffered Saline (PBS) and digested into cell suspensions suspended in fresh complete medium and for flow cytometry analyzed. Fluorescence excitation wavelength and collection wavelength described in the paper.

2.8. In vivo mice imaging

In vivo imaging of mice performed on Perkinelmer IVIS Lumina XRMS Series III In Vivo Imaging System. Fluorescence imaging detection gain remains constant throughout the experimental process.

2.8.1. Nude mice in vivo imaging

BALB/c nude mice (female, 4 weeks old), intratumoral injection of probe Cy-OAcrr or NEM, after 1 h, the mice were anesthetized with isoflurane gas and then performed in vivo fluorescence imaging. Imaging includes bright field, fluorescence and X-ray. Fluorescence excitation wavelength and collection wavelength described in the paper.

2.8.2. Orthotopic lung cancer mice in vivo imaging

FVB mice (male, 6 weeks old) were administered Cy-OAcrr through the trachea, after 15 min and 30 min, the mice were anesthetized with isoflurane gas then in vivo imaging. Imaging includes bright field, fluorescence and X-ray. Lung tissues imaging were performed after the end of in vivo imaging. Fluorescence excitation wavelength and collection wavelength described in the paper.

Scheme 1. Synthetic route of probe Cy-OAcrr.

a) sodium acetate, anhydrous DMF, 50 °C 12 h, 87%; b) acryloyl chloride, anhydrous CH2Cl2, 0 °C 1 h, then 25 °C 12 h, 91%.

Scheme 2. Molecular structure and proposed mechanism of Cy-OAcrr for the ratiometric detection of Cys.

2.9. Ethics statement

All experimental procedures were conducted in conformity with institutional guidelines for the care and use of laboratory animals, and protocols were approved by the Institutional Animal Care and Use Committee in Binzhou Medical University, Yantai, China. Approval Number: No. BZ2014-102R.

3. Results and discussion

3.1. Design and synthesis of Cy-OAcrr

Inspired by the design strategy from Yoon et al. [55] work, we strive to synthesis a ratiometric NIR fluorescent probe for the detection of Cys in cells and in vivo. As shown in Scheme 2, Ketone cyanine (Keto-Cy) is chosen as the fluorescence signal transducer. The chemical modification of a fluorescence modulator on the meso-position of this cyanine fluorophore will result in the change of the conjugated π-electron system, which has been proved to be an efficient approach for dominating fluorescence emission profile changes [55,59]. Moreover, the substituent on N-atom of indoline is changed to ethyl. This tiny difference of molecular structure can reduce thermal effects of NIR light, which may be beneficial for the protection of cells for the non-invasive tests [60]. α, β-Unsaturated acrylate (Acrr) is selected as the fluorescence modulator because it could discriminate Cys, Hcy, and GSH via an unique adduct/intramolecular cyclization [61–65]. Our efforts finally result in a ratiometric NIR probe Cy-OAcrr for the specific detection of Cys over Hcy and GSH. The synthesis routes of Cy-OAcrr was described in Scheme 1. Ketone-type cyanine dye (Keto-Cy) was prepared from the substitution and hydrolysis reaction between heptamethine cyanine and sodium acetate in anhydrous N,N-dimethylformamide [66]. The incorporation of acryloyl chloride into Keto-Cy in anhydrous dichloromethane would afford the final probe Cy-OAcrr. The related chemical structures were identified via 1H NMR, 13C NMR, and HRMS. The detection mechanism of the probe Cy-OAcrr was shown in Scheme 2.

3.2. Spectral properties and selectivity of Cy-OAcrr

The absorption and fluorescence spectra of Cy-OAcrr were examined under simulated physiological conditions (10 mM HEPES buffer, pH 7.4, 37 °C). UV-vis spectra of Cy-OAcrr were detected after addition of Cys in HEPES (Fig. S3a). When treated with increasing concentrations of Cys (0–20 µM), the absorption peak centered at 769 nm (ε_769 nm = 1.0 × 10^5 M^−1 cm^−1) decreased, and a new absorption peak centered at 505 nm (ε_505 nm = 1.8 × 10^4 M^−1 cm^−1) emerge, respectively. The isosbestic point was at 595 nm. The color of the solution turned from green into red. The obvious color change indicated that Cy-OAcrr potentially served as a naked-eye probe for Cys. As expected, there was a large hypsochromic spectral shift in emission profiles (Fig. 1a, b). As the
Cys concentration gradient increased, the fluorescence emission of Cy-Oacr at 794 nm (Φ = 0.076) dramatically decreased, and finally resulted in the fluorescence emission of Keto-Cy at 635 nm (Φ = 0.35). The spectral shift in emission profiles could provide an efficient ratiometric approach for Cys detection in cells and in vivo. There was a linear concentration-dependent ratiometric fluorescent response with Cy-Oacr towards Cys ranging from 0 to 20 μM, and the calibration curve was Lg(F794 nm/F635 nm) = 5.95 × 10^{-2} [Cys] μM − 0.67 with a linear fitting constant r = 0.9918 (Fig. 1c). The limit of detection was calculated as 0.09 μM (3σ/k) under the experimental conditions. The results indicated that our probe could be used to detect intracellular Cys qualitatively and quantitatively. The time-dependent spectral changes of the probe showed that the release of fluoresphore Keto-Cy was very fast and complete within 30 min (Fig. S3b).

As shown in Fig. 1d, when reacted with potential interference species, the probe only offered a very high level of ratiometric fluorescence emission towards Cys. Other reactive biothiols, such as GSH and Hcy displayed quite a little of fluorescence increase but far lower than that of Cys. The reason was attributed to that Hcy and GSH might efficient in the initial nucleophilic attack at the α, β-unsaturated acrylate of Cy-Oacr but inefficient in the subsequent intramolecular cyclization. Mixture of Cy-Oacr with amino acid (aspartic acid, glutamic acid, glycine, and lysine), bovine serum albumin (BSA), reactive oxygen/nitrogen species and reactive species (NO, H2O2, and HOCl), anions and cations (Ca2+, Fe2+, Zn2+, Br−, and Cl−) at the corresponding physiological concentrations did not induce any detectable fluorescence changes. Additionally, the addition of Cys (0.2 mM) to the mixture of Hcy (0.2 mM) and GSH (5 mM) also induced a strong increase of fluorescence ratio as the mixture of the probe with Cys only (Fig. S3c). These results showed that the probe Cy-Oacr was a ratiometric NIR fluorescent probe that had promising potential for the highly selective detection of Cys. Our probe should be a suitable candidate for Cys detection in the biological systems.

### 3.3. Cytotoxicity and mitochondrial localization

We chose human lung cancer cell lines (A549 cell line, H1650 cell line, and PC9 cell line) as cell test models, because the cancer cells have higher levels of Cys than normal cells [4]. Cys might be a potential biomarker for tumor diagnosis. Before cell experiments, we first investigated the cytotoxicity of Cy-Oacr. The three cell lines were cultured in 96-well plates and incubated with gradient change concentration of Cy-Oacr (0–100 μM) for 24 h. Then Cell Counting Kit-8 (CCK-8) solution was added and incubated for 30 min. The absorbance value at 450 nm was measured using a microplate reader. The results displayed that the probe Cy-Oacr had low cytotoxicity and the IC50 values of A549 cells, H1650 cells, and PC9 cells were 120.12 μM, 122.19 μM and, 125.48 μM, respectively.

Mitochondria play critical roles in many physiological processes including reactive oxygen species (ROS) generation, apoptosis initiation, and cellular proliferation regulation [67]. The functional and regulatory mitochondrial Cys modifications can modulate protein activities to cell stress [68]. But the damage and dysfunction of mitochondrion will result in cancer and multiple neurodegenerative diseases [69]. Given the important physiological functions of Cys in mitochondria, the effective imaging of mitochondrial Cys enabled us to better understand its important roles in the process of cancer development. The lipophilic iminium cation in the backbone of Cy-Oacr would allow the probe preferentially accumulating in mitochondria. In order to confirm the mitochondria localization ability of the probe, we employed two commonly used commercially mitochondria-specific dyes, Mito-Tracker Green and Rhodamine123, to carry out the colocalization experiments using confocal laser scanning microscope. The spectrally separated images acquired from the four dyes were estimated using Image-Pro Plus software (Fig. 2). As illustrated in Fig. 2, the fluorescence images of the probe were well merged with the fluorescence images of Mito-Tracker Green and Rhodamine123, respectively. The Pearson’s correlation coefficients between our probe and Mito-Tracker Green in A549, H1650, and PC9 cells were 0.90, 0.94, and 0.97, respectively. The Pearson’s correlation coefficients between our probe and Rhodamine123 in A549, H1650, and PC9 cells were 0.93, 0.95 and 0.91, respectively. We also performed intensity correlation analysis of the dyads (between the probe and Mito-Tracker Green/Rhodamine 123, respectively). The containing analysis was based on counting the intensity of stain color-pair for each pixel, which demonstrated the intensity distribution of the two colocalization dyes. As shown in Fig. 2 (correlation), only the contain Cy-Oacr against mitochondrial dyes offered highly correlated plots. It should be noted that the fluorescence images of the probe were acquired from the fluorescence collection window from 610 to 700 nm, where the fluorescence emission belonged to the Keto-Cy fluorophore. We performed lysosome containing experiments to further verify the localization capability of our probe Cy-Oacr in mitochondria. The A549 cell line, H1650 cell line, and PC9 cell line were incubated with the probe Cy-Oacr for 30 min, LysoTracker Green DNA-26 for 30 min, and nuclear dye Hoechst 33,342 for 30 min, respectively. As shown in Fig. S8, probe Cy-Oacr had poor localization in lysosome. That was, our probe Cy-Oacr could exactly react with Cys in mitochondria. The above results verified that probe Cy-Oacr possessed excellent mitochondrial-targeting ability and could be used to detect mitochondrial Cys in living cells.

### 3.4. Detection of cysteine levels in different cell lines

We next investigated whether the probe Cy-Oacr could quickly respond to intracellular Cys. The A549, H1650 and PC9 cells were incubated with 10 μM Cy-Oacr for 10 min, then the cells were washed with corresponding fresh cell culture mediums. As shown in Fig. 3a, the time-dependent ratiometric changes of the probe demonstrated that the detection process was very fast and completed within 30 min. The probe Cy-Oacr could also be utilized for Cys detection via flow
cytometry analysis. As shown in Fig. 3b–d the fluorescence intensities for flow cytometry analysis were collected at 0 min and 30 min. The result displayed that the fluorescence intensity of Channel 1 decreased while the fluorescence intensity of Channel 2 increased. The probe reacted completely within 30 min. The results were highly consistent with the ratio values in Fig. 3a and e. The results suggested that Cy-OAcr could quickly penetrate into cells and rapidly react with Cys in mitochondria.

We further performed four control cell assays to verify the selectivity of the probe Cy-OAcr towards Cys in cells. As illustrated in Fig. 4, the cells in group a were treated with N-ethylmaleimide (NEM) for 30 min to deplete all the intracellular biothiols, then added Cy-OAcr incubated for 30 min. The cells in group b, c, and d were preincubated with exogenous Cys, Glutathione monoethyl ester (GSH-MEE), and Hcy for 30 min. After washed with the corresponding fresh cell culture medium for three times, these cells were continuously cultured in bovine serum-free cell culture medium and were incubated with Cy-OAcr for 30 min. There no ratio signal were observed in Fig. 4a due to the intracellular Cys had been scavenged by NEM. Because the addition of exogenous Cys supplemented the intracellular Cys concentration (Fig. 4b), fluorescent images from Channel 2 emitted strong red fluorescence, and the fluorescence ratio signals were significantly increased compared to the absence of exogenous Cys (Fig. 3a). However, the addition of exogenous GSH-MEE did not contribute to the remarkable changes in fluorescence (Fig. 4c). The similar results were obtained in the group d pretreated with exogenous Hcy (Fig. 4d). The mean ratios of group a, b, c, and d were displayed in Fig. 4e–h. On the basis of our findings, we confirmed that our probe Cy-OAcr could selectively utilize to detect intracellular Cys.

3.5. Detection of cysteine in tumor-bearing mice

Since the probe Cy-OAcr had successfully applied to the selective detection of Cys in cells, we next explored whether the probe could be used to detect Cys in vivo with the remarkable NIR optical properties. In vivo imaging of small animals system was used for the detection of Cys in tumor-bearing mice. The male nude mice (4 weeks old, ~20 g) in Fig. 5a were divided into six groups with bearing A549, H1650, PC9 cell lines tumor, respectively. After intratumorally injected Cy-OAcr for 1 h, the animals were subjected to in vivo imaging system for Cys detection. The fluorescent images in Fig. 5a were constructed from two fluorescence collection windows: Channel 1: $\lambda_{ex} = 740$ nm, $\lambda_{em} = 750$–830 nm; Channel 2: $\lambda_{ex} = 520$ nm, $\lambda_{em} = 580$–660 nm. Channel 2 (group a, b, and c) offered strong fluorescence from tumor lesion of the mice as the probe had detected Cys. However, when the mice in group I, II, and III were intratumorally preinjected NEM for to deplete all the biothiols in tumor lesion, the later injection of Cy-OAcr provided a dramatically decreasing intensity fluorescence image in
Channel 2 compared to those mice without pretreatment of NEM. Fig. 5b illustrated the ratio of mean number of photons for each group shown in Fig. 5a. The above results confirmed that the NIR probe Cy-OAcr well behaved as the desirable candidate for detecting and imaging of Cys in complicated living organisms.

3.6. Detection of cysteine in orthotopic lung cancer mice

To verify the potential contribution of our probe Cy-OAcr for clinical auxiliary diagnosis, we performed further assays for the detection of Cys in the orthotopic lung cancer mice models. We used urethane to stimulate FVB mice to establish the orthotopic lung cancer mice model. The FVB mice were intraperitoneally injected with 8% urethane (dissolved in saline) at a dose of 0.01 ml g$^{-1}$ twice a week. The whole
process consumed five weeks. Then it would take three months for the development of orthotopic lung cancer lesions. All the mice in Fig. 6a were administrated Cy-OAcr (10 μM, 200 μl, in DMSO : saline = 1 : 99, v/v) through the tracheal spray. After 30 min, the mice were anesthetized with isoflurane for in vivo imaging. Normal mice in group a were as control. The orthotopic lung cancer mice model in group b were three months, while the mice model in group c were four months. The results showed that the fluorescence intensity of Channel 2 in the lung cancer mice models was higher than that of the normal group, indicating the higher levels of Cys in the lung cancer mice models than in the normal mice. In the orthotopic lung cancer model group b and c, The fluorescence intensity of group c in Channel 2 was stronger than that
of in group b. The time-dependent quantization of fluorescence ratio was shown in Fig. 6c. In order to enable the diagnostic ability of our probe Cy-OAcr, all the mice were sacrificed after the performance of in vivo imaging. The isolated lungs were further underwent fluorescence imaging in vitro (Fig. 6b). The results were consistent with the results of in vivo imaging. Cancer lesions could be found in lung (arrows in bright microscopy (Fig. 6d). The fluorescence intensities of the model group in Channel 2 were higher than the normal group. These results demonstrated that Cys concentration in tumor tissue were higher than that in normal tissue. We performed hematoxylin-eosin (H&E) pathological staining to verify the lung tissue (Fig. 6e). The result of H&E illustrated that nuclear atypia of group c was more clear than group b. This result also well explained why the fluorescence intensity in Channel 2 of group c was stronger than that of group b. Our results indicated that the probe Cy-OAcr had excellent ability to detect Cys in vivo. Given the high levels of Cys in tumor tissues, this new ratiometric NIR fluorescent probe had potentiality in the clinical auxiliary diagnosis of cancer.

4. Conclusions

In conclusion, based on previously research, we synthesize a ratiometric NIR fluorescent probe Cy-OAcr for highly selective detection of Cys from other structurally and functionally similar biotoxins, such as GHS and Hcy. The rapid discrimination of Cys is based on an unique adduct/intramolecular cyclization reaction, which results in remarkable spectra shifts via modulating the conjugated π-electron system of cyanine fluorophore. The probe Cy-OAcr has low cytotoxicity and excellent membrane permeability. The probe preferentially accumulates within mitochondria and is capable of evaluating the levels of mitochondrial Cys in living cancer cell lines. Taking advantage of Cy-OAcr, we have achieved the detection and imaging of Cys in three tumor-bearing mice models. Moreover, this ratiometric NIR fluorescent probe has been successfully applied for bioimaging Cys in orthotopic lung cancer mice models. The results demonstrate that our probe has high potential for the clinical auxiliary diagnosis of cancer.

Acknowledgements

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Appendix A. Supplementary data

Supplementary material related to this article can be found in the online version, at doi:https://doi.org/10.1016/j.snb.2018.11.056.

References

[1] D.P. Jones, Y.M. Go, C.L. Anderson, T.R. Ziegler, Jr J.M. Kinkade, W.G. Kirlin, Cysteine/cystine couple is a newly recognized node in the circuitry for biologic fields of Fig. 6b). Obviously, cancer lesions emitted higher fluorescence than the normal lung tissue in Channel 2. The confocal images of the above-described fresh lung tissue sections were taken from confocal microscopy (Fig. 6d). The fluorescence intensities of the model group in Channel 2 were higher than the normal group. These results demonstrated that Cys concentration in tumor tissue were higher than that in normal tissue. We performed hematoxylin-eosin (H&E) pathological staining to verify the lung tissue (Fig. 6e). The result of H&E illustrated that nuclear atypia of group c was more clear than group b. This result also well explained why the fluorescence intensity in Channel 2 of group c was stronger than that of group b. Our results indicated that the probe Cy-OAcr had excellent ability to detect Cys in vivo. Given the high levels of Cys in tumor tissues, this new ratiometric NIR fluorescent probe had potentiality in the clinical auxiliary diagnosis of cancer.

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