Biomaterials 35 (2014) 4157–4167

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

A fluorescent probe to detect thiol-containing amino acids in solid tumors

Wen Xiu Ren [a,1], Jiyou Han [b,1], Tuhin Pradhan [a], Ja-Yun Lim [b], Jae Hong Lee [a], Jaehun Lee [b], Jong-Hoon Kim [b,**], Jong Seung Kim [a,*]

[a] Department of Chemistry, Korea University, Seoul 136-701, Republic of Korea
[b] Division of Biotechnology, Laboratory of Stem Cells and Tissue Regeneration, College of Life Sciences & Biotechnology, Korea University, Seoul 136-713, Republic of Korea

ARTICLE INFO

Article history:
Received 4 December 2013
Accepted 22 January 2014
Available online 11 February 2014

Keywords:
GSH measurement
Ratiometric fluorescence
Tumor tissue
Blood
Hepatocellular carcinoma

ABSTRACT

Early detecting of cancer is critical to provide proper treatment and to improve survival of patients. Here, we reported a highly sensitive ratiometric (yellow emission (550 nm) to blue emission (496 nm)) fluorescent probe 1 developed for detection of thiol-containing amino acids. This probe successfully eliminates interference from background autofluorescence, and discriminates between human carcinoma and normal cells by detecting intracellular thiol levels in living cells ($P < 0.05$). Furthermore, the ability of the probe to identify growing tumors by measuring GSH in the tissues as well as in the fresh blood of tumor xenograft mice. Additionally, the ratio of the emission intensity at two different wavelengths can provide quantitative analysis of glutathione (GSH) in the living systems. It suggests that it represents a promising prognostic and diagnostic marker, with extensive and simple potential clinical applications.

1. Introduction

The development of reliable and simple tools for predicting the appearance of cancer tissue is an emerging focus in preclinical and clinical oncology research. Several studies have suggested that elevated glutathione (GSH) concentration in malignant tissues and blood in cancer patients may be a meaningful surrogate marker, not only for tumor progression, but also for poor responsiveness to chemotherapy [1–3]. GSH is the most abundant intra-cellular non-protein thiol, and plays a pivotal role in cellular homeostasis by acting as a redox regulator to maintain the cellular reducing environment [4–6]. As a consequence, through its maintenance of reduced levels of glutaredoxin or thioredoxin, which are required for DNA synthesis, GSH is involved in differentiation, proliferation, and apoptosis in normal and malignant cells [7,8]. Oxidative stress arising from disturbances in GSH homeostasis is involved in the etiology and progression of many human pathophysiological conditions including cancer [9]. GSH levels in mammalian tissues normally range between 1 and 10 mM, with the highest levels seen in the liver [4,10]. Liver cancer is the third foremost cause of cancer-related deaths worldwide, due to its high rate of distant metastases and therapy-resistant local recurrences (as high as 50% within 2 years) after surgical resection or chemoembolization [11,12]. Resistance to chemotherapy is therefore one of the most important clinical problems in the treatment of liver cancer. Thus, the development of sensitive and accurate fluorescent probes to detect fluctuations of intracellular GSH concentration, in living carcinoma cells as well as in vivo solid tumors models, is desirable. Significant effort has therefore been devoted to developing biological assays to detect and measure GSH concentration in clinical and preclinical biomatrices. These efforts have been hampered, however, by the autoxidation, degradation, and instability of GSH in biomatrices and in lysed cells or tissues.

To address these issues, activatable fluorescent probes that can accurately recognize and standardize the intra-cellular GSH have been developed [3,13]. Nonetheless, the quantification of GSH in biomatrices remains a complicated goal due to high background interference from other proteins, as well as cross-reactivity with cysteine (Cys) and homocysteine (Hcy), which have similar structures to GSH, but different physiological functions [14–17]. Although the strong nucleophilicity of the thiolate group has been leveraged to develop fluorescence sensors for the detection of thiols [18–23], most probes have comparable specificity for Cys, Hcy, and GSH, and only a small number of probes can discriminate

* Corresponding author. Fax: +82 2 3290 3121.
** Corresponding author. Fax: +82 2 3290 3007.
E-mail addresses: jhkim@korea.ac.kr (J.-H. Kim), jongskim@korea.ac.kr (J.S. Kim).
[1] These authors contributed equally to this work.

0142-9612/$ – see front matter © 2014 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biomaterials.2014.01.055

between these moieties in bioenvironments [19,22]. As a consequence, there is a pressing need for the development of selective fluorescent probes with high sensitivity and selectivity, and low cytotoxicity for the detection of GSH in living cells and tissues.

2. Design and synthesis

2.1. Design strategy of probe

Chromenone derivatives are promising receptors for Michael nucleophilic addition [24–26]. In addition, the introduction of specific functional groups into sensors to increase electrostatic attraction between bio-thiols and sensors has proven to be good strategies for enhancing the selectivity of thiol detection [23,27]. Accordingly, we designed and synthesized a new fluorescence probe **1** for the identification of biological thiols by attaching a pyridinium moiety to the chromenone skeleton via a vinyl linker (Scheme 1). In the presence of thiols, the ring opening of the chromene moiety induced by Michael addition will block the chromenone fluorescence channel and activate on the phenylpyridylvinylene channel, leading to ratiometric changes in fluorescence. This effectively reduces interference from autofluorescence in various biomatrices, resulting in a high signal/background ratio.

2.2. Synthesis

As shown in Scheme 2, probe **1** was synthesized from 2-hydroxy-5-(hydroxymethyl)benzaldehyde [28] in 3 steps with good yields.

Compound **2**: To a solution of 2-hydroxy-5-(hydroxymethyl) benzaldehyde (600 mg, 4 mmol) in THF (3 ml), 1.5 equiv. 2-cyclohexen-1-one (576 mg, 6 mmol) and 1.5 equiv. imidazole (408 mg, 6 mmol) was added. After mixed with deionized water (3 ml), the mixture was stirred at room temperature. After completion of the reaction (as monitored by TLC), the final mixture was treated with 1 M HCl (20 ml) and extracted with ethyl acetate. The organic layer was dried by Na_2SO_4 and concentrated under vacuum. Flash chromatographic purification on silica gel (Hexane/EtOAc = 100%–30%) afforded **2** (460 mg, 50% yield) as a yellow solid. ^{1}H NMR ($CDCl_3$) δ (ppm): δ 1.64–1.75 (m, 1H), 1.95–2.11 (m, 2H), 2.33–2.61 (m, 3H), 4.60 (s, 1H), 4.95–4.99 (m, 1H), 6.86 (d, J = 8.3 Hz, 1H), 7.21 (s, 1H), 7.24–7.27 (m, 1H), 7.39 (s, 1H); ^{13}C NMR ($CDCl_3$) δ (ppm): 18.14, 29.81, 39.02, 64.78, 74.91, 116.32, 122.28, 128.76, 130.76, 131.27, 131.65, 134.96, 155.56, 197.86; ESI-MS: m/z = 229.05 $[M-H]^-$.

Compound **3**: To a suspension of pyridinium chlorochromate (PCC) (646 mg, 3 mmol) in dichloromethane (50 ml), **2** (460 mg, 2 mmol) was added, and then the reaction mixture was stirred at room temperature and monitored by TLC. After completion of the reaction, silica gel was added to adsorb the sticky byproduct. The mixture was filtered and washed with water. The organic layer was dried by Na_2SO_4 and concentrated under vacuum. Flash chromatographic purification on silica gel (Hexane/EtOAc = 100%–70%) afforded **3** (460 mg, 80% yield) as a yellow solid. ^{1}H NMR ($CDCl_3$) δ (ppm): δ 1.68–1.80 (m, 1H), 2.00–2.18 (m, 2H), 2.37–2.67 (m, 3H), 5.11–5.16 (m, 1H), 6.99 (d, J = 8.4 Hz, 1H), 7.42 (d, J = 2.2 Hz, 1H), 7.73 (d, J = 1.9 Hz, 1H), 7.79 (dd, J = 8.4 Hz, 1H), 9.88 (s, 1H); ^{13}C NMR ($CDCl_3$) δ (ppm): 18.11, 29.81, 39.01, 75.69, 117.03, 122.29, 130.14, 131.23, 131.46, 131.87, 133.69, 160.86, 190.48, 197.24; ESI-MS: m/z = 227.05 $[M-H]^-$.

Compound **1**:1-ethyl-4-methylpyridinium iodide was prepared according to the literature [29]. **3** (82 mg, 0.36 mmol) and 1-ethyl-4-methylpyridinium iodide (76 mg, 0.3 mmol) were mixed in ethanol (20 ml), and then piperidine (0.02 ml) was added to the solution. The reaction mixture was refluxed with stirring for 1 h and then evaporated in vacuo. The resulting solid was dissolved in CH_2Cl_2, and the organic layer was washed three times with water, dried over anhydrous $MgSO_4$, and evaporated in vacuo. The residue was purified by column chromatography on silica (from MeOH/CH_2Cl_2 = 5%–8%) to give **1** (56 mg, 40%) as a light brown solid. ^{1}H NMR ($CDCl_3$): δ (ppm): 1.70 (t, J = 7.3 Hz, 3H), 2.08–2.16 (m, 1H), 2.35–2.45 (m, 2H), 2.49–2.64 (m, 3H), 4.81 (q, J = 7.2 Hz, 2H), 5.05–5.09 (m, 1H), 6.93 (d, J = 8.5 Hz, 1H), 7.07 (d, J = 16.2 Hz, 1H), 7.37–7.38 (m, 1H), 7.49 (d, J = 1.6 Hz, 1H), 7.58–7.61 (m, 1H), 7.68 (d, J = 8.2 Hz, 1H), 8.06 (d, J = 6.6 Hz, 2H), 9.00 (d, J = 6.6 Hz, 2H). ^{13}C NMR ($CDCl_3$): δ (ppm): 17.14, 18.11, 29.82, 39.05, 56.94, 75.30, 117.41, 122.92, 123.90, 127.46, 128.13, 130.08, 130.36, 131.47, 132.68, 141.06, 144.09, 154.54, 157.19, 197.58. HRMS (FAB) m/z calcd for $C_{22}H_{22}NO_2^+$ [M]: 332.1651. Found 332.1651.

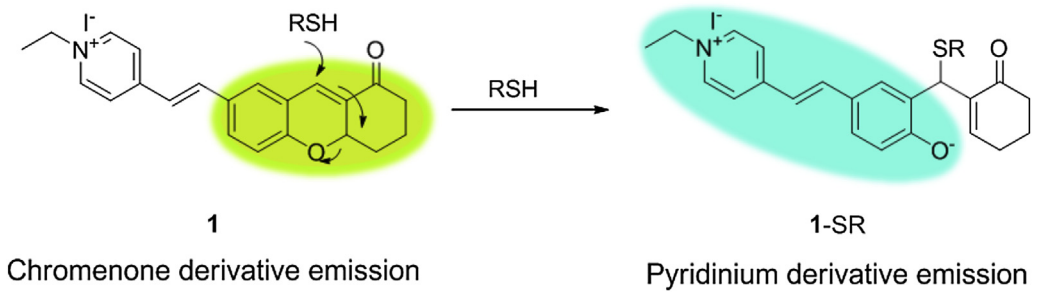
3. Materials and methods

3.1. General information and materials

All fluorescence and UV/Vis absorption spectra were recorded with Shimadzu RF-5301PC and Agilent 8453 spectrophotometers, respectively. All ^{1}H and ^{13}C NMR spectra were collected in $CDCl_3$ or DMSO*d-6* on a Varian 300 and 400 MHz spectrometer. All chemical shifts are reported in ppm value using the peak of residual proton signals of TMS as an internal reference. ESI mass spectral analyses were carried out using LC/MS-2020 Series (Shimadzu). The fluorescence imaging of cells and tissues was performed with a confocal laser scanning microscope (Carl-Zeiss LSM 5 Exciter, Oberko, Germany). Signal quantification of fluorescent intensity in blood samples was measured by using a Perkin Elmer Victor 2 1420 multi-label microplate readers (Perkin Elmer, Turku, Finland). All analytes were purchased from Aldrich and used as received. All solvents were analytical reagents from Duksan Pure Chemical Co., Ltd. All DMSO for spectra detection was HPLC reagent grade, without fluorescent impurity; H_2O was deionized.

3.2. Spectroscopic data

Stock solutions (1.0 mM) of the biologically relevant analytes [amino acids (Cys, Hcy, GSH, Ala, Arg, Asn, Asp, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Tau, Thr, Trp, Tyr, Val), metals (K^+, Ca^{2+}, Mg^{2+}, Na^+, Zn^{2+}), and reactive oxygen species (H_2O_2)] were prepared in twice distilled water. Stock solutions of **1** (1 mM) were prepared in DMSO (dimethyl sulfoxide). For all measurements of fluorescence spectra, excitation was at 372 nm with all the excitation slit widths at 5 nm, and emission at 5 nm. All UV/Vis and fluorescence titration experiments were performed using 20 μM and 10 μM of **1** in PBS solutions (pH = 7.4, 10 mM) with varying concentrations of analytes at room temperature.



Scheme 1. Design of thiol specific probe **1** for ratiometric fluorescence change.