Sensors and Actuators B 231 (2016) 615–623

Contents lists available at ScienceDirect

Sensors and Actuators B: Chemical

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

A highly selective fluorescent probe for sensing activities of catechol-*O*-methyltransferase in complex biological samples

Xing-Kai Qian[a,b,1], Ping Wang[b,1], Yang-Liu Xia[b], Tong-Yi Dou[b], Qiang Jin[b], Dan-Dan Wang[b], Da-Cheng Hao[c], Xiao-Lin Bi[a,*], Guang-Bo Ge[b,*], Ling Yang[b,*]

[a] *Institute of Cancer Stem Cell, Cancer Center; Department of Biological Sciences, College of Basic Medical Sciences, Dalian Medical University, Dalian 116044, China*
[b] *Laboratory of Pharmaceutical Resource Discovery, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116023, China*
[c] *Dalian Jiaotong University, Dalian 116028, China*

ARTICLE INFO

Article history:
Received 4 February 2016
Received in revised form 11 March 2016
Accepted 17 March 2016
Available online 18 March 2016

Keywords:
Catechol-*O*-methyltransferase
Fluorescent probe
7,8-Dihydroxy-4-methylcoumarin
High-throughput detection
Bioimaging

ABSTRACT

Catechol-*O*-methyltransferase (COMT), one of the most important phase II drug metabolizing enzymes, plays important roles in the metabolism of endogenous and xenobiotic catechols. In this study, a highly selective fluorescent probe for sensing activities of catechol-*O*-methyltransferase in complex biological samples was discovered and well characterized. Under physiological conditions, COMT selectively catalyzes the conversion of the probe (7,8-dihydroxy-4-methylcoumarin, **DHMC**) to 7-hydroxy-8-methoxy-4-methylcoumarin (**HMMC**), which brings a strong turn-on fluorescence signal at 520 nm. The probe substrate has been used for monitoring the real activities of COMT in complex biological samples, as well as for rapid screening of potential COMT inhibitors which are useful in the treatment of Parkinson's diseases Furthermore, the probe has been successfully used to monitor endogenous COMT in living cells for the first time, and the results demonstrate that **DHMC** is cell membrane permeable and low toxic to the cells. All these features of **DHMC** suggested that this probe holds great promise for COMT-related regulation and inhibition assays in drug discovery, as well as for further investigation on the biological functions of COMT in living cells.

1. Introduction

Catechol *O*-methyltransferase (COMT EC 2.1.1.6) is a metal ions-dependent enzyme that catalyzes the transfer of a methyl group from common methyl donor S-adenosyl-L-methionine (AdoMet or SAM) to one of the catecholic hydroxyls [1,2]. COMT plays a key role in the bio-activation of a wide range of endogenous compounds (such as dopamine, noradrenaline, and adrenaline) and exogenous compounds (such as protocatechuic acid, caffeic acid, 5,6-dihydroxyindoles, and flavonoids) [3]. Clinically, COMT participates in the metabolism of many neurotransmitters and its inhibitors play a critical role in the management of central nervous system (CNS) disorders [4–8], such as Parkinson's disease [9,10]. Estrogen catechol metabolites are carcinogenic [11,12], which might be involved in both their estrogenicity and the oxidative metabolism to genotoxic quinones [13,14]. COMT is an important phase II enzyme that catalyzes *O*-methylation of catechols to block their estrogenicity and further metabolism [14].

In humans, COMT is a strictly intracellular protein that has soluble (s-COMT) and membrane-bound (MB-COMT) isoforms, both of which are products of the same gene (COMT, Comt) [15,16]. S-COMT has a high enzymatic capacity, but it binds catechol substrates with much lower affinity than MB-COMT [17]. COMT is widely distributed throughout the organs of the body. The highest COMT activity in humans is in the liver, and s-COMT activity is prominent [18]. COMT protein in vertebrates appears mostly in a soluble form (as s-COMT), and only a minor fraction is in the particular form (as MB-COMT) [3,17].

In the past decades, several methods for the assay of COMT have been reported in sample solutions from homogenates [19–22]. Methods using spectrophotometry [23–25] and fluorime-

Abbreviations: COMT, catechol-*O*-methyltransferase; SAM, S-adenosyl-L-methionine; DHMC, 7,8-dihydroxy-4-methylcoumarin; HMMC, 7-hydroxy-8-methoxy-4-methylcoumarin; 7-MHMC, 7-methoxy-8-hydroxy-4-methylcoumarin; hLS9, human liver S9.

* Corresponding authors.
E-mail addresses: bxl@dlum.cn (X.-L. Bi), geguangbo@dicp.ac.cn (G.-B. Ge), yling@dicp.ac.cn (L. Yang).

[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.snb.2016.03.074
0925-4005/

try [26–30] seem to be most effective. The currently used probe metabolites are often detected by liquid chromatography-tandem mass spectrometry (LC–MS/MS) or high performance liquid chromatography (HPLC). Okada et al. reported the first fluorimetric method for the assay of COMT by measuring the amount of vanillin formed enzymatically from 3,4-dihydroxybenzaldehyde [29]. Nohta et al. [30] provided the first method for the assay of COMT in erythrocytes that did not use the radioisotopic substrate or cofactor in HPLC with the fluorescence detector. The limit of detection for m- and *p*-methylated products was 3 pmol per assay tube (60 fmol per injection volume of 20 μl). The ratio of *m*- and *p*-methylated products was 0.54 [30]. COMTs in both methods were detected and quantified in HPLC or LC–MS/MS. Kurkela et al. developed a 96-well microplate assay to identify inhibitors of human s-COMT, using aesculetin as the substrate. The methylation of aesculetin to scopoletin is measured directly in a reaction mixture in which SAM serves as the methyl group donor. The reaction is quantified fluorometrically [31].

The present study aimed to develop a novel fluorescent probe for selectively sensing the enzyme activity of COMT in complex biological systems. 7,8-Dihydroxycoumarin was selected as the basic fluorophore as it possesses a polycyclic aromatic skeleton and has desirable photophysical properties, such as large Stokes shifts and high photostability [32]. Modulation of the electron donating ability of C-8 catechol group would trigger significant changes in emission spectra, which makes 7,8-dihydroxycoumarin and its derivatives desirable fluorescence probes [33]. In our preliminary study, a series of 4-substituted 7,8-dihydroxycoumarin derivatives were synthesized to evaluate their potential as decent substrates of COMT (Fig. S1). The initial screening revealed that 4-methoxy-7,8-dihydroxycoumarin (**DHMC**) [34] displayed well reactivity towards COMT. These findings prompted us to further characterize the selectivity and sensitivity of this COMT probe, and explore the feasibility and practicability of its use in complex biological systems.

2. Experimental

2.1. Materials and instrumentations

All chemicals were commercial products of analytical grade. Human liver S9 was obtained from Research Institute for Liver Diseases (RILD, Shanghai, China). BCA Protein Assay Kit was obtained from Beyotime (Beijing, China), human COMT ELISA kit was from eBioscience, Inc. (California, USA). MCF-7, HepG2, LNCaP, and U87-MG cells were purchased from the Committee on Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). All other reagents, fine chemicals, and LC solvents, with the highest grade commercially available, were from J&K Chemical Ltd. (Shanghai, China), Tedia (Fairfield, OH, USA), and Sigma (St. Louis, MO, USA). ^{1}H NMR and ^{13}C NMR spectra were recorded using Bruker vance II 400 MHz spectrometer with chemical shifts reported as ppm (DMSO-d_6; TMS as internal standard). High-resolution mass (HRMS) data were measured on Fourier transform ion cyclotron resonance mass spectrometer (LTQ Orbitrap XL). Fluorescence emission/excitation spectra were measured on Synergy H1Hybrid Multi-Mode Microplate Reader (BioTek). The methylation supernatants were determined using a Shimadzu UFLC system coupled with a mass spectrometer (Shimadzu 2010 EV, Japan) or fluorescence detector (Shimadzu RF 20A, Japan). The fluorescence images of cells were taken using an inverted fluorescence microscope (Leica, Wetzlar, Germany) with an objective lens (×10 or ×40). The stock solution of **DHMC** (1 mM) was prepared in DMSO and stored at −80 °C for future use. Human liver S9 samples and the recombinant human s-COMT (2.5 mg/ml) were prepared in the phosphate buffer (pH 7.4) and stored at −80 °C.

2.2. Bacterial expression and purification of recombinant human s-COMT

The plasmid sample of human s-COMT (108 V) in the Novagen pET22b(+) vector with a C-terminal histidine tag was a generous gift from Prof. Judith P. Klinman (University of California, Berkeley). The expression and purification of the enzyme were performed following the procedures reported by Zhang and Klinman [35], with minor changes. The recombinant s-COMT was transformed and expressed in *Escherichia coli* BL21 (DE3) cells. Transformed BL21 (DE3) cells were induced with 1 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) when the absorbance at 600 nm was around 0.6, and then grown at 37 °C overnight while shaking at 200 rpm. The cells were harvested *via* centrifugation at 5000*g* for 20 min at 4 °C, and then re-suspended in 100 mM tris–HCl (pH 8), 0.3 M NaCl, 1 mM EDTA, 20% glycerol (v/v), 10 mM β-mercaptoethanol (ME), 5 mM $MgCl_2$ with lysozyme (1 mg/ml) and incubated on ice for 30 min. The suspended solution was sonicated, adding phenylmethylsulfonyl fluoride (PMSF, 0.1 M) in advance. After lysis, the cells were centrifuged at 20,000*g* for 20 min at 4 °C. The clear supernatant was applied to a pre-equilibrated HisTrap™ HP metal affinity column (GE Healthcare). The column was washed with at least 50 column volumes of wash buffer (100 mM Na_2PO_4 (pH 8.0), 0.4 M NaCl, 20% glycerol (v/v), 10 mM β-ME, 5 mM $MgCl_2$, and 20 mM imidazole). S-COMT was collected by using elution buffer (100 mM Na_2PO_4 (pH 7.4), 0.25 M NaCl, 20% glycerol (v/v), 5 mM β-ME, 5 mM $MgCl_2$, and 200 mM imidazole). Protein samples were concentrated and desalted using a 15 ml Amicon® Ultra centrifugal filter, and stored at −80 °C until further use. Protein concentrations were calculated using the Bradford assay.

2.3. Synthesis of O-methylated derivatives of *DHMC*

The structure of the metabolite formed *via* COMT was confirmed by comparison of LC retention times, UV and mass spectra organic synthesis of two *O*-methylated derivatives of **DHMC** including 8-hydroxy-7-methoxy-4-methylcoumarin (**7-MHMC**) and 7-hydroxy-8-methoxy-4-methylcoumarin (**HMMC**), which were synthesized by the following schemes.

8-hydroxy-7-methoxy-4-methylcoumarin (**7-MHMC**): To a mixture of 3-methoxybenzene-1,2-diol (0.5 g, 3.5 mmol) and ethyl acetoacetate (0.95 ml, 7.0 mmol) was added drop-wise perchloric acid (3.0 ml) at room temperature and stirred for 6 h. After completion of the reaction as indicated by TLC, the reaction mixture was poured slowly into a mixture of ice-water (50 ml) with stirring. The resultant suspension was filtered and the collected solid was washed with water and dried, then the crude compound was recrystallized from methanol to afford 7-MHMC as light white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.38 (s, 3H, CH_3), 3.89 (s, 3H, OCH_3), 6.20 (s, 1H, COCH=C), 7.03 (d, J = 8.0 Hz, 1H, Ar-H), 7.21 (d, J = 8.0 Hz, 1H, Ar-H), 9.38 (s, 1H, OH). ^{1}C NMR (400 MHz, DMSO-d_6) δ: 18.7, 61.1, 110.8, 113.4, 113.5, 120.9, 134.7, 148.1, 154.1, 154.3, 160.4 (Fig. S2); ESI–MS: M = 206, found 205 $[M-H]^-$. HRMS (ESI positive) calcd for [M−H] 207.0579, found 207.0651 (Fig. S4).

7-hydroxy-8-methoxy-4-methylcoumarin (**HMMC**): To the solution of 7,8-dihydroxy-4-methylcoumarin (0.8 g, 4.1 mmol) in DMF was added NaH, 60% suspension in oil (0.41 g, 10.3 mmol) at 0° C under dry argon and stirred for 0.5 h methyl iodide (0.33 ml, 4.9 mmol) was added drop-wise to the reaction mixture at 5 °C, maintaining the temperature at 0–5° C for 2 h until the reaction was completed, as indicated by TLC. The reaction mixture was poured into water-ice and acidified with 2 N hydrochloric acid. The reaction mixture was extracted with ethyl acetate (50 ml × 3).