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A highly selective ratiometric fluorescent probe for in vitro monitoring and cellular imaging of human carboxylesterase 1

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ABSTRACT

A new ratiometric fluorescent probe derived from 2-(2-hydroxy-3-methoxyphenyl) benzothiazole (HMBT) has been developed for selective monitoring of human carboxylesterase 1 (hCE1). The probe is designed by introducing benzoyl moiety to HMBT. The prepared latent spectroscopic probe **1** displays satisfying stability under physiological pH conditions with very low background signal. Both the reaction phynotyping and chemical inhibition assays demonstrated that hCE1 mediated the specific cleavage of the carboxylic ester bond of probe **1** in human biological samples. The release of HMBT leads to a remarkable red-shifted emission in fluorescence spectrum (120 nm large emission shift). Furthermore, human cell-based assays show that probe **1** is cell membrane permeable, and it can be used for bioassay and cellular imaging of hCE1 activity in HepG2 cells. These findings lead to the development of a simple and sensitive fluorescent method for measurement of hCE1 activity in vitro or in living cells, in the presence of additional enzymes or endogenous compounds.

1. Introduction

Carboxylesterases (E.C.3.1.1.1) are members of α/β-hydrolase fold proteins found in mammals (Redinbo and Potter, 2005; Satoh and Hosokawa, 1998). In human, most carboxylesterases have been identified belonging to carboxylesterase 1 (hCE1) and carboxylesterase 2 (hCE2) (Wang et al., 2011). As a broad-spectrum serine hydrolase, hCE1 catalyzes the hydrolysis of a large number of structurally diverse of endogenous and exogenous substrates including fatty acids, environmental toxins and drugs (Brzezinski et al., 1997; Redinbo et al., 2003; Shi et al., 2006; Sun et al., 2004; Tang et al., 2006; Wheelock et al., 2008; Zhang et al., 1999; Zhu et al., 2009b). Besides the key roles in drug metabolism and toxin detoxication, hCE1 also participates in several physiological processes including lipid homeostasis, testosterone synthesis, retinol metabolism, trafficking and retention of proteins in the endoplasmic reticulum (Bencharit et al., 2006; Crow et al., 2008; Harrison, 1998; Igarashi et al., 2010; Quiroga et al., 2012; Xu et al., in press). It has been reported that the deficiency of hCE1 may lead to several diseases, such as atherosclerosis, obesity, hepatic steatosis, hyperlipidemia, and hepatocellular carcinoma (Friedrichsen et al., 2013; Na et al., 2009; Quiroga et al., 2012; Sekiya et al., 2009; Xie et al., 2010). As the most abundant carboxylesterase distributed in human, hCE1 is primarily expressed in liver, with lesser amounts in intestine, kidney, lung, testes, heart, monocytes and macrophages (Sanghani et al., 2009; Satoh et al., 2002). The enzyme shares 47% sequence identity with hCE2 which is primarily expressed in the gastrointestinal tract but at a lower level in liver (Redinbo et al., 2003; Ross and Crow, 2007; Taketani et al., 2007). Notably, the expression and the function of human carboxylesterases can be altered by the genetic, environmental, and pathological factors (Hagihara et al., 2009; Nemoda et al., 2009; Ross et al., 2012; Tang et al., 2006; Yoshimura et al., 2008). A large interindividual variability of hCE1 have been detected in mRNA, protein level and hydrolytic activity (Yang et al., 2009), these influences greatly limit the understanding of the precise contribution of hCE1 in its related biological processes. Furthermore, many therapeutic drugs and herbal constitutes display strong inhibitory effects toward the catalytic activity of hCE1, which may lead to potential drug–drug interactions or drug–herbal interactions (Crow et al., 2008; Hatfield et al., 2013; Liu et al., 2010; Rhoades et al., 2012;

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Shi et al., 2006; Zhu et al., 2010). All of these studies have subsequently prompted interest in devising selective and sensitive methods to monitor the real function of hCE1 in biological samples.

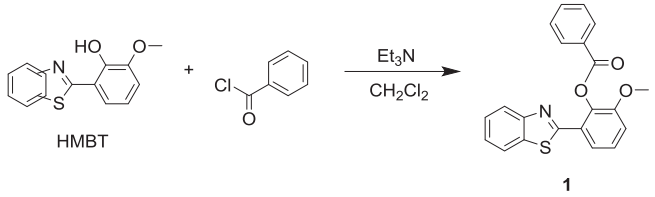
Up to date, several methods including antibody-based (immunochemical) methods and mass spectrometry-based proteomic techniques, have been developed for hCE1 quantification in biological samples (Sato et al., 2012; Zhu et al., 2009b). However, these methods are relatively complicated, time-consuming, and limited by some stringent requirements (such as high qualified operators or expensive instruments). Furthermore, these methods only can evaluate the protein levels of hCE1 rather than its real activity. Therefore, it is necessary to develop a more practice method for rapid and selective monitoring the real activity of hCE1. Recently, small molecule fluorescent probes for selective monitoring a given target in biological samples are more attractive, due to their inherent advantages, such as highly specific and sensitive, non-destructive, easily-conducted, as well as applicable to high-throughput screening (Choi et al., 2013; Cui et al., 2010; Demchenko and Callis, 2010; Li et al., 2014; Zhu et al., 2014). Although several fluorescence probes for various carboxylesterases including hCE2 have been reported (Hakamata et al., 2011; Kim et al., 2007; Wang et al., 2011; Zhang et al., 2012), the fluorescent probe for selective detection of hCE1 has not been reported yet.

In this paper, we developed a HMBT-based ratiometric fluorescent probe, 2-(2-benzoyloxy-3-methoxyphenyl) benzothiazole (**1**, see Scheme 1) for highly selective detection of hCE1. The probe is designed by introducing benzoyl as a quenching and reacting moiety to HMBT. The observed fluorescence blue-shift of **1** can be ascribed to the inhibition of the ESIPT mechanism by the benzoyl moiety. Upon reaction with hCE1, the carboxylic ester bond is cleaved by the enzyme after the specific substrate recognition, leading to the release of HMBT. As a result, the reaction solution displays a remarkable fluorescence spectrum change, which provides the basis for the sensitive detection of hCE1 activity. Moreover, probe **1** also displays good cell membrane permeability and can be used for imaging the hCE1 activity in living HepG2 cells.

2. Materials and methods

2.1. Materials and instruments

All the starting materials for synthesis were commercially available and used as received. Paraoxonase-1 (PON1, v13111203) and paraoxonase-2 (PON2, v13111202) were obtained from Bioworld (MN, USA). Acetylcholinesterase (AChE, C1682), butyrylcholinesterase (BChE, B4186) and human serum albumin (HSA, 018k7540) were all obtained from Sigma (MO, USA). Three recombinant hCEs isoforms including hCE-1b (10980), hCE-1c (10989) and hCE-2 (11269) were purchased from BD Biosciences (USA). ^{1}H-NMR and ^{13}C-NMR spectra were recorded using Bruker Avance II (400 MHz) spectrometer with chemical shifts reported as ppm (in $CDCl_3$; TMS as internal standard). High-resolution mass data were measured on Fourier transform ion cyclotron resonance mass spectrometer (LTQ Orbitrap XL). Fluorescence emission/excitation spectra were measured on Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek). LC–DAD–ESI–MS measurements were performed on a Shimadzu UFLC system coupled with a diode array detector and a mass spectrometer (Shimadzu 2010 EV, Japan). Stock solution of **1** (0.5 mM) was prepared in DMSO and stored at −80 °C for future use. Stock solutions (5 mg/mL) of enzymes were prepared in phosphate buffer (pH 7.4).



Scheme 1. Synthesis of probe **1**.

2.2. Synthesis of probe *1*

To a solution of 0.5 mmol HMBT and Et_3N (0.625 mmol) in 10 mL of CH_2Cl_2, benzoyl chloride (0.6 mmol, mixed with 5 mL of CH_2Cl_2) was added dropwise at 0 °C in 30 min. After stirring for 1 h, the mixture was warmed to room temperature and stirred overnight. The solvent was removed in vacuum, and the residual solid was purified by chromatography (silica gel, ethyl acetate–hexane as eluent, 1:3, v/v) to afford 77.5 mg (yield 45.8%) of **1** as a white solid. The structure of **1** was confirmed by ^{1}H NMR, ^{13}C NMR and HRMS spectroscopy (see Figs. S1–S3). ^{1}H NMR (400 MHz, $CDCl_3$): 8.34 (2H, d, J=8.0 Hz), 8.05 (1H, dd, J=8.1 Hz), 8.00 (1H, d, J=8.2 Hz), 7.80 (1H, d, J=8.0 Hz), 7.70 (1H, dd, J=10.6 Hz), 7.58 (2H, t, J=7.8 Hz), 7.48–7.31 (3H, m), 7.14 (1H, dd, J=8.2 Hz), 3.87 (3H, s). ^{13}C NMR (100 MHz, $CDCl_3$): 56.34, 114.12, 121.31, 121.39, 123.32, 125.24, 126.22, 126.82, 127.62, 128.69, 129.37, 130.78, 133.78, 135.57, 138.43, 152.05, 152.67, 162.18, 164.20. HRMS (ESI positive) calcd for **1** $[M+H]^+$: 362.0851 and found: 362.0840.

2.3. General procedure for characterization of the selectivity of probe *1*

Measurements of the activity of carboxylesterases or other hydrolytic enzymes were carried out in 100 mM PBS (pH 7.4) according to the following procedure. Incubation mixtures, with a total volume of 0.2 mL, consisted of 100 mM PBS (pH 7.4), and appropriate amount of human liver microsomes or other mentioned enzymes protein, were mixed gently. Then reactions were started by addition of the stock solution of probe **1**, with the final concentration of DMSO in reaction mixture not exceeding 1% (v/v, without any interference in the catalytic activity, see Fig. S9). After incubation at 37 °C in a shaking water bath, the reaction was terminated by the addition of ice-cold acetonitrile (equal volume of incubation mixture, 0.2 mL). The mixtures were then centrifuged at 20,000g for 10 min. Aliquots of supernatant were taken for further fluorescence analysis (Gain=100). Control incubations without enzyme sources were carried out to ensure that metabolites formation was enzyme dependent.

2.4. Chemical inhibition assays

In order to verify the involved human enzyme for the hydrolytic biotransformation of probe **1**, inhibition assays were performed using a series of selective esterase inhibitors as described previously, including bis(p-nitrophenyl) phosphate (BNPP) for carboxylesterases (Hatfield and Potter, 2011), loperamide (LPA) for hCE2 (Quinney et al., 2005), and tetraisopropyl pyrophosphoramide (iso-OMPA) for butyrylcholinesterase (BChE) (Giacobini, 2003). Briefly, in a shaking water bath, probe **1** was incubated in pooled HLMs in the absence (control) or presence of known selective esterase inhibitors. Each inhibitor (100 μM) was pre-incubated within the reaction mixtures (0.2 mL, total volume) containing 0.1 M PBS (pH 7.4) and HLMs (10 μg/mL) at 37 °C for 10 min, then the reaction was started by the addition of **1** (10 μM, final concentration), other procedures including termination step and sample preparation were depicted as described previously.