A highly selective near-infrared fluorescent probe for carboxylesterase 2 and its bioimaging applications in living cells and animals

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ABSTRACT

A near-infrared fluorescent probe (**DDAB**) for highly selective and sensitive detection of carboxylesterase 2 (CE2) has been designed, synthesized, and systematically studied both *in vitro* and *in vivo*. Upon addition of CE2, the ester bond of **DDAB** could be rapidly cleaved and then release a near-infrared (NIR) fluorophore **DDAO**, which brings a remarkable yellow-to-blue color change and strong NIR fluorescence emission in physiological solutions. The newly developed probe exhibits excellent properties including good specificity, ultrahigh sensitivity and high imaging resolution. Moreover, **DDAB** has been applied to measure the real activities of CE2 in complex biological samples, as well as to screen CE2 inhibitors by using tissue preparations as the enzymes sources. The probe has also been successfully used to detect endogenous CE2 in living cells and *in vivo* for the first time, and the results demonstrate that such detection is highly reliable. All these prominent features of **DDAB** make it holds great promise for further investigation on CE2-associated biological process and for exploring the physiological functions of CE2 in living systems.

Keywords: NIR fluorescent probe; Carboxylesterase 2; Enzymatic activity; Specific detection; Bioimaging

1. Introduction

Carboxylesterases (CEs) are important phase I drug-metabolizing enzymes localized within the lumen of the endoplasmic reticulum (ER) of many mammal tissues (Redinbo and Potter 2005). CEs play a key role in the detoxification or metabolic activation of various ester-drugs, environmental toxicants, and carcinogens (Ross and Crow 2007; Satoh and Hosokawa 2006). In human, the majority of CEs have been identified belong to the CE1 or CE2 family. These two isoforms share approximately 40%–50% amino acid sequence identity, and exhibit differential tissue distribution and substrate specificity (Imai et al. 2006). Generally, CE1 hydrolyzes substrates with small alcoholic groups and large acyl groups, such as oseltamivir and clopidogrel (Zhu et al. 2013). In contrast to CE1, CE2 mainly recognizes substrates with large alcohol and small acyl group, such as capecitabine and irinotecan. Furthermore, CE1 is highly expressed in the liver, lung and brain, and considerably reduced expression in the gastrointestinal tract, whereas CE2 is present predominantly in the intestine and liver (Satoh et al. 2002; Zhang et al. 2002).

In recent years, CE2 has gained increasing attention in the treatment of cancer. It has been reported that CE2 plays an important role in the metabolic activation of anticancer prodrugs including irinotecan, capecitabine, and LY2334737 (the prodrug of gemcitabine) (Hatfield et al. 2011; Pratt et al. 2013; Walko and Lindley 2005). Rational design of CE2-activated prodrugs is considered as a practical strategy for modification of potent anticancer agents or those agents with narrow therapeutic index. However, the expression and function of CE2 can be greatly affected by many genetic or environmental factors, which may strongly affect the release of active metabolite(s) (Yang et al. 2009). Thus, the efficient monitoring and detection of CE2 in different biological systems (such as tissues and cancer cell lines) is of great importance for drug discovery and clinical practice. On the other hand, although CE2 has broad and overlapping xenobiotic substrate specificities, its endogenous substrates and CE2-associated biological functions *in vivo* have not been well characterized (Ross and Crow 2007). Thus it is necessary to explore the real functions of endogenous CE2 in living cells or animals.

In recent years, several sensitive fluorescent sensors have been reported and used to monitor the enzymatic activities of CE2 in tissue and cell preparations (Feng et al. 2014a, 2015b; Liu et al. 2014; Wang et al. 2011). However, all these probes have a severe limitation on *in vivo* applications to detect CE2 in living systems, since their emission wavelengths are less than 630 nm,

which could be easily interfered by matrix autofluorescence and cannot gain good tissue penetration. Given that the real function of a given enzyme *in vivo* is more likely different from that *in vitro* (Evans and Cravatt 2006), the development of novel tools for exploring the functions of endogenous CE2 in *ex vivo* or *in vivo* models is highly desirable. It is well-known that near-infrared (NIR) fluorescent probes can overcome the above mentioned shortcomings on account of its minimal photodamage to biological samples, good tissue penetration, and minimal interference from background autofluorescence in complex biological systems (Hilderbrand and Weissleder 2010; Hu et al. 2013; Li et al. 2015; Yin et al. 2015; Yuan et al. 2013). Unfortunately, to the best of our knowledge, none of the NIR fluorescent probes for *in vivo* monitoring CE2 have been reported. Therefore, the development of a new readily available NIR fluorescent probe for rapid, selective, and sensitive detection of CE2 in living cells and living animals is urgently demanded.

Inspired by the substrate preference and catalytic properties of CE2, a novel colorimetric near-infrared fluorescence probe (**DDAB**) derived from 1, 3-dichloro-9, 9-dimethyl-9H-acridin-2(7)-one (**DDAO**) has been designed and developed for the selective and sensitive sensing CE2 in complex biological samples. **DDAB** shows emission at NIR region, an ideal profile for penetration through thicker biological specimens, and exhibits rapid, selective, and sensitive fluorescence turn-on response to CE2 with a 57-fold fluorescence enhancement. Furthermore, **DDAB** could permeate through the cell membrane and thus was applicable for CE2 detection in living cells. In addition, we have successfully applied this probe to live mouse and its tissues such as the liver, small intestine, lung, heart, and spleen.

2. Experimental

2.1 Materials and instrumentations

Paraoxonases (EC 3.1.1.2) including PON1 (v13111203) and PON2 (v13111202) were obtained from bio-world, α 1-Acid Glycoprotein (AAG, G9885), α -chymotrypsin (EC 3.4.21.1, α -CT, C4129), carbomic Anhydrase I (EC 4.2.1.1, CA, C4396), pepsin (EC 3.4.23.1, P6887), trypsin (EC 3.4.21.4, T1426), acetylcholinesterase (EC 3.1.1.7, AChE, C1682), butyrylcholinesterase (EC 3.1.1.8, BChE, B4186), and human serum albumin (HSA, 018k7540) were all obtained from Sigma-Aldrich. Carboxylesterases (EC 3.1.1.1) including CE1 and CE2, pooled human liver microsomes (HLM), pooled human kidney microsomes (HKM), and pooled human intestine microsomes (HIM) were received from RILD (Shanghai, China). Bis-p-nitrophenyl phosphate

(BNPP), loperamide (LPA) fluorescein diacetate (**FD**), and fluorescein (**F**) were purchased from TCI (Tokyo, Japan). Ethylene diamine tetraacetic acid (EDTA) and huperzine A (HA) were obtained from J&K Chemical Ltd. (Beijing, China). SKOV-3 and HepG2 cell lines were purchased from the American Type Culture Collection (Teddington, Middlesex, UK). **HL-7702 cell lines (human normal liver cells) were purchased from the Committee on type Culture Collection of Chinese Academy of Sciences.** Stock solution of **DDAB** (10 mM) was prepared in DMSO and stored at -80 °C until use. Stock solutions (2 mg/mL) of enzymes were prepared in phosphate buffer (pH=7.4, 100 mM). All fluorescence tests for CE2 detection were performed on Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek). NMR spectra were measured with a 400 MHz Bruker spectrometer set TMS as reference for ¹H and ¹³C NMR. Accurate mass correction is measured with LTQ Orbitrap XL. The hydrolysis supernatants were determined by a Shimadzu UFLC system coupled with a diode array detector and a mass spectrometer (Shimadzu 2010 EV, Japan).

2.2 Synthesis of **DDAB**

To a solution of 0.25 mmol **DDAO** and Et₃N (0.3125 mmol) in 10 mL of CH₂Cl₂, benzoyl chloride (0.3 mmol, mixed with 5 mL of CH₂Cl₂) was added dropwise at 0 °C in 30 min (Scheme S1). After stirring at this temperature for 1 h, the mixture was warmed to room temperature and stirred overnight. The solvent was removed in vacuo, and the residual solid was purified by chromatography (silica gel, EtOAc–hexane as eluent, 1: 5, v/v) to afford 36.5 mg (35.2%) of **DDAB**. The structures of **DDAB** were confirmed by ¹H NMR, ¹³C NMR and HRMS spectroscopy (Fig. S13-S15), and the data are as follows: ¹H NMR (400 MHz, CDCl₃) δ 8.22 (d, *J* = 7.4 Hz, 2H), 7.73 (d, *J* = 8.5 Hz, 1H), 7.71 – 7.63 (m, 2H), 7.55 (t, *J* = 7.7 Hz, 2H), 7.39 (d, *J* = 2.3 Hz, 1H), 7.28 (dd, *J* = 8.8, 2.6 Hz, 1H), 1.92 (s, 6H). ¹³C NMR (100 MHz, CDCl₃) δ 173.19, 164.66, 153.67, 149.86, 140.37, 139.50, 138.66, 137.41, 135.60, 134.08, 133.17, 130.28, 128.85, 121.67, 120.03, 77.08, 76.74, 76.70, 39.15, 26.72. HRMS (ESI positive) calcd for [M+H]⁺ 412.0502, found 412.0504.

2.3 General procedure for monitoring carboxylesterase activity

On the basis of the procedure following, all the measurements of carboxylesterase activity were carried out in 100 mM phosphate buffer (PBS, pH = 7.4) containing 1% DMSO. With a

total incubation volume of 0.2 mL, incubation mixtures that contained PBS and mentioned enzymes protein or microsomes were mixed gently. Then a proper amount of **DDAB** was added to start the reactions. After incubation at 37 °C in a shaking water bath for 15 min, an equal volume of ice-cold acetonitrile was added to terminate the reactions. The mixtures were then centrifuged for 5 min at 20 000g. Aliquots of supernatant were taken for further fluorescence analysis (Gain 100). All assays were performed in duplicates.

2.4 Cell culture and fluorescence imaging

Cells were grown in corresponding culture medium (SKOV-3 and HepG2 cell were cultured in MEM/EBSS medium, HL-7702 cells were grown in RPMI-1640 medium) supplemented with fetal bovine serum (FBS) of 10% in an atmosphere of 5% CO₂ and 95% air at 37 °C. Cells were seeding at a density of 10⁵ cells per dish (Φ 20 mm) and incubated overnight. After washed twice with FBS-free culture medium, the adherent cells were incubated with/without 100 μM BNPP (preparing in FBS-free) for 60 min at 37 °C in 5% CO₂ incubator. For detection of endogenous CE2, the living cells were co-incubated with **DDAB** (20 μM) and Hoechst 33342 at 37 °C for another 20 min. After washed with PBS (pH 7.4) for three times, cells were imaged on confocal microscope (Olympus, FV1000, Japan).

2.5 Fluorescent imaging in living mice

Nude mice, 6-8 weeks old, were obtained from Laboratory Animal Center of Dalian Medical University. All the animal experiments were carried out in accordance with the guidelines issued by the Ethical Committee of Dalian Medical University. Mice were divided into two groups, in the experimental group, the mouse was given an intraperitoneal injection of **DDAB** (150 μL, 200 μM in PBS buffer). In the control group, mouse was injected with equal amount of PBS and imaging under the same conditions. The mouse images were taken after incubation for 30 min by using a NightOWL II LB983 small animal *in vivo* imaging system containing a sensitive Charge Coupled Device (CCD) camera, with an excitation laser of 630 nm and an emission filter of 700 ±10 nm.

2.6 Western blot analysis.

The protein concentrations of HepG2 cells S9 fractions were measured using BCA protein assay. Then 50 µg protein of per sample was loaded into per well on a 10 % polyacrylamide gel. After electrophoresis, the separated proteins were transferred onto Sequi-Blot PVDF membranes (Bio-Rad, Hercules, CA) in transfer buffer [192 mM glycine, 25 mM Tris-base, pH 8.3, 20% (v/v) methanol]. Membranes were blocked with 5% nonfat dried milk in TBST buffer [20 mM Tris-HCl, pH 8.0, 50 mM NaCl, 0.1% (v/v) Tween-20] for 1 h at room temperature. Membranes were then incubated with specific anti-human carboxylesterase 2 antibody (Abcam, Cambridge, UK) overnight at 4 °C and further incubated with HRP-conjugated anti-antibody (Abcam, Cambridge, UK) for 1 h at room temperature after washed with TBST buffer for three times. Calnexin (GenScript, New Jersey, USA) was used to establish the internal standard. The blots were washed three times with TBST buffer, and the immunoreactive protein bands were detected by enhanced chemiluminescence (Vilber Lourmat, Marne-la-Vallée, FRA).

3. Results and discussion

3.1. Synthesis and detection mechanism

To get a CE2-specific fluorescent probe with emission in the NIR region for potential *in vivo* applications, **DDAO** was selected as the basic fluorophore because of its desirable spectroscopic properties, good photostability, low toxicity and feasibility in structural modification (Labruere et al. 2012; Tung et al. 2004; Warther et al. 2010). The fluorescence properties of **DDAO** could be modulated *via* the principle of intramolecular charge transfer (ICT) process by incorporating an aromatic group (Gao et al. 2015). Thus, a benzoyl group was intentionally incorporated into **DDAO** *via* esterification. After being esterified with benzoyl group, the fluorescence of **DDAO** was completely quenched by blocking the ICT progress. The structure and the recognition mechanism of **DDAB** for CE2 detection are shown in Scheme 1. The details of the synthetic procedure of **DDAB**, as well as structural characterization by HRMS, ¹H NMR, and ¹³C NMR are described in Supporting Information (Fig. S13-S15).

Scheme 1 The structure of DDAB and its fluorescence response towards CE2.

3.2. Spectral properties of **DDAB** towards **CE2**

The sensing ability of **DDAB** to **CE2** was firstly investigated under physiological conditions (pH 7.4 at 37 °C). **DDAB** was very stable in PBS at 37 °C without any hydrolysate detected following 15 min incubation. Upon addition of **CE2**, **DDAB** could be easily hydrolyzed and formed a single metabolite **DDAO** (Fig. S1), which triggered a dramatic increase in the fluorescence emission at 662 nm (Fig. 1B). Thus, **DDAO** could serve as a “turn-on” NIR fluorescent probe for **CE2**. In agreement with the fluorescence switch-on response, treating **DDAB** with **CE2** could also induce a dramatic change in its UV absorption profile. **DDAB** itself exhibited a major absorption band centered at 430 nm, whereas **CE2** treatment resulted in the disappearance of this band and the appearance of a new band centered at 646 nm (Fig. 1A). Such changes in absorption bring the solution from yellow to blue, indicating that **DDAB** can serve as a “naked-eye” indicator for **CE2**. The effects of pH on the fluorescence intensity of **DDAB** and its metabolite **DDAO** was also investigated and depicted in Fig. S2, **DDAB** was stable in a wide pH range of 4–9, while **DDAO** was stable in a narrow pH range of 7–9. Hence, the PBS at pH 7.4 was used for further investigations. In addition, photostability of **DDAB** was investigated, due to it was very important for NIR fluorescent detection both *in vitro* and *in vivo*. As shown in Fig. S3, the fluorescence intensity of **DDAO** remained at 95% following illumination under a UV lamp at 365 nm for 2 h, indicating good photostability of the probe under the detection conditions. These results indicated that **DDAB** could be used as a colorimetric and NIR turn-on fluorescent probe with little background fluorescence for rapid detection of **CE2** in aqueous solution under physiological conditions.

Fig. 1 Spectral changes of **DDAB** during its reaction with human CE2 in PBS (pH=7.4, 100 mM) at 37 °C. UV-vis absorption (A) and emission spectra changes (B) of **DDAB** (10 μ M) in the presence of CE2 (5 μ g/mL). λ_{ex} =600 nm. Color and emission color (under a 365 nm light) changes are inserted, respectively.

3.3. Selectivity and sensitivity of **DDAB**

To explore the applicability of **DDAB** for sensing CE2 in complex biological samples, the selectivity was carefully investigated. As shown in Fig. 2A, significant fluorescence enhancement was observed upon addition of CE2 (5 μ g/mL), while no significant fluorescence enhancement was observed upon addition of other hydrolases, including CE1, cholinesterases (AChE and BChE), paraoxonases (PON1 and PON2), α -chymotrypsin (α -CT), carbonic anhydrase I (CA), Pepsin, Trypsin, α 1-acid glycoprotein (α 1-AG), bovine serum albumin (BSA), and human serum albumin (HSA). To further investigate the selectivity of **DDAB** towards CE2, chemical inhibition assays were conducted in human tissue preparations, by using a series of selective inhibitors against esterases. We could see from Fig. 2B that both bis (4-nitrophenyl) phosphate (BNPP, a potent inhibitor of CEs) and loperamide (LPA, a selective inhibitor of CE2) could strongly inhibit **DDAB** hydrolysis in human liver microsomes (HLM), human intestine microsomes (HIM) and human kidney microsomes (HKM), while other selective inhibitors displayed negligible inhibitory effects on this biotransformation. The fluorescence response of **DDAB** to various biologically relevant small molecules was also investigated to further investigate its anti-interference ability in complex biological system. As expected, the fluorescence intensity of **DDAB** towards CE2 could not be influenced by addition of common biological metallic ions or amino acids in animal

tissues or fluids (Fig. S4). These results strongly suggested that **DDAB** hydrolysis was selectively catalyzed by CE2, and it could serve as an efficient tool for measuring the real activity of CE2 in complex biological matrixes.

Fig. 2 (A) Fluorescence responses of **DDAB** (10 μM) towards various species of enzymes at 37 $^{\circ}\text{C}$ for 30 min. CE1 and CE2 (5 $\mu\text{g}/\text{mL}$), carbonic anhydrase I (CA, 5 $\mu\text{g}/\text{mL}$), a-chymotrypsin (a-CT, 5 $\mu\text{g}/\text{mL}$), pepsin (5 $\mu\text{g}/\text{mL}$), trypsin (5 $\mu\text{g}/\text{mL}$), a1-Acid Glycoprotein (AAG, 5 $\mu\text{g}/\text{mL}$), bovine serum albumin (BSA, 50 $\mu\text{g}/\text{mL}$), human serum albumin (HSA, 50 $\mu\text{g}/\text{mL}$), acetylcholinesterase (AChE, 5 $\mu\text{g}/\text{mL}$), butyrylcholinesterase (BChE, 20 U/L), paraoxonase (PON1 and PON2, 5 $\mu\text{g}/\text{mL}$). (B) Inhibitory effects of specific inhibitors of human esterases on **DDAB** (10 μM) hydrolysis in HLM (4 $\mu\text{g}/\text{mL}$), HIM (4 $\mu\text{g}/\text{mL}$) HKM (4 $\mu\text{g}/\text{mL}$) and CE2 (2 $\mu\text{g}/\text{mL}$). Data are shown as mean $\pm$ SD. The spectra were measured in phosphate buffer (PBS)-acetonitrile (v/v=1:1, pH 7.4). λ_{ex} = 600 nm.

After made the high selectivity of **DDAB** for CE2 clear, we turned our attention to its sensitivity for CE2. To this end, the fluorescence signal changes of **DDAB** upon addition a cascade of CE2 (0-10 $\mu\text{g}/\text{mL}$) were monitored under same conditions. **DDAB** itself was quite stable under the tested conditions, upon addition of CE2 with different concentrations, the fluorescence intensity gradually increased at 662 nm (Fig. S5a). In addition, the red fluorescence intensity was found linearly increased when CE2 was progressively added from 0 to 7 $\mu\text{g}/\text{mL}$ ($R^2=0.997$, Fig. S5b). The limit of detection (3 S/N) of **DDAB** for CE2 was estimated as 0.07 $\mu\text{g}/\text{mL}$. Such high sensitivity could be attributed to its NIR emission with little interference by matrix auto-

fluorescence. Therefore, these results indicated that **DDAB** could not only be used to quantitatively detect CE2 *in vitro*, but also hold great promise for sensing trace CE2 in living systems.

3.4. Enzymatic kinetics of CE2-mediated **DDAB** hydrolysis

It is well known that the kinetic behavior was crucial for the quantitative applications of the activity-based probes (Ge et al. 2013; Dai et al. 2015; Jin et al. 2015). In this study, the kinetic analysis of **DDAB** hydrolysis was well characterized in different enzyme sources including human recombinant CE2, HLM (human liver microsomes), HIM (human intestine microsomes) and HKM (human kidney microsomes). As shown in Table 1 and Fig. S6, **DDAB** hydrolysis in both recombinant CE2 and human tissue preparations displayed very high affinity ($K_m < 2 \mu\text{M}$) and good reactivity ($K_{cat}/K_m > 6 \text{ mL/min/mg protein}$). Notably, the K_m values of **DDAB** hydrolysis in different human tissue preparations were very similar to that in recombinant human CE2, implying that CE2 was the predominant enzyme responsible for **DDAB** hydrolysis in human tissues. These results suggested that **DDAB** hydrolysis displayed excellent selectivity towards CE2 and ideal kinetic behaviors, which are of great importance for quantitative detection of enzymatic activities of CE2 in complex biological systems.

Table 1. Kinetic parameters for DDAB hydrolysis in different enzyme sources.

Enzyme sources	$V_{\max}$ (nmol/min/mg)	K_m (μM)	CL_{int} (mL/min/mg)
HIM	8.88±0.32	0.95±0.13	9.35
HLM	16.5±0.74	1.83±0.24	9.02
HKM	9.38±0.59	1.43±0.14	6.56
CE2	20.40±0.66	1.92±0.31	10.63

3.5. Biomedical applications

Encouraged by these above findings, we next investigated the utility of **DDAB** for screening of CE2 inhibitors by using human tissues preparations as enzyme sources. As shown in Fig. S7, both the inhibition profiles and the IC_{50} values of the two known inhibitors (BNPP and LPA) towards **DDAB** hydrolysis in both HLM and HIM were similar to those in CE2. These findings further confirmed that **DDAB** could be used as a highly selective fluorescent probe of CE2, for high throughput screening of CE2 modulators by using tissue preparations as enzyme sources.

The practical application of **DDAB** for measuring the real activities of CE2 in human biological samples was then investigated. As depicted in Fig. S8, **DDAB** was used to determinate CE2 activities in a panel of twelve HLMs. About 3-fold difference (12 to 36 nmol/min/mg protein) in hydrolytic activity was observed among individuals, which was in accordance with previous literature reported variability in enzymatic activity assayed by other probe substrates (Ross et al. 2012; Xu et al. 2002). Moreover, a strong correlation with a high coefficient parameter ($R^2=0.96$, $P < 0.0001$) was gained between the hydrolytic rates of **DDAB** and the hydrolytic rates of **FD** (a commercially available probe of CE2). These outcomes strongly indicated that **DDAB** could be employed to measure the real activity of CE2 in a mixture of multiple enzymes, and the quantification was highly reliable.

3.6. Bioimaging of CE2 in living cells

Next, we attempted to monitor intracellular CE2 by using this NIR fluorescence probe. As show in Fig. 3, following incubation of SKOV-3 cells with **DDAB** (20 μ M) at 37 $^{\circ}$ C for 20 min, the cells started to show strong red fluorescence. In sharp contrast, when the cells were pretreated with BNPP (100 μ M), the cells showed dim red fluorescence. Encouraged by the above results of imaging endogenous CE2 in SKOV-3 cells, we use the probe to monitor endogenous CE2 in HepG2 cells. The similar fluorescence imaging results were gained when the probe was incubated with HepG2 cells with or without BNPP (Fig. S9a, d). We subsequently conducted chemical inhibition assays in cell lysates, and found that the pretreatment of BNPP or LPA in lysates could remarkably reduce the hydrolysis of **DDAB** (Fig. S11). In addition, we found that pretreatment of 5-FU (a reported inducer of CE2) (Xiao et al. 2013) could increase intracellular hydrolysis of **DDAB**, which led to a significant red fluorescence enhancement (Fig. S9b, c). To specify the induction of CE2, we tested the expression of CE2 in both mRNA and protein levels. As shown in Fig. S10, 5-FU induced CE2 mRNA level in a dose-dependent manner and proportionally increased protein expression of CE2, which were consistent with the elevated CE2 activities. Additionally, to compare the efficiency of the probe in healthy cells, we use **DDAB** to imaging CE2 in HL-7702 cells. To our delight, similar fluorescence images were afforded (Fig. 4). Furthermore, MTT assays showed that cell viability for cells was more than 90 % even though 50 μ M **DDAB** was added for 48 h (Fig. S12), suggesting the low toxicity of the probe. Taken to-

gether, our results demonstrated that **DDAB** was cell membrane permeable and capable of sensing endogenous CE2 in living cells.

Fig. 3 Confocal fluorescence images of SKOV-3 cells co-stained with **DDAB** and **Hoechst 33342**. (a-d) Cells treated with **DDAB** (20 μ M) and **Hoechst 33342** (2 μ M) at 37 $^{\circ}$ C for 20 min; (e-g) Cells pretreated with BNPP (100 μ M) and further stained with **DDAB** (20 μ M) and **Hoechst 33342** (2 μ M). (b, e) Fluorescence images from **Hoechst 33342** (nuclear staining); (c, f) images from **DDAB**; (d, g) overlay of the blue and red channels. Scale bars= 30 μ m.

Fig. 4 Confocal fluorescence images of HL-7702 cells costained with **DDAB** and **Hoechst 33342** (nuclear staining). (a) Cells treated with Hoechst 33342 (2 μ M)), (b-e) Cells treated with **DDAB** (20 μ M) and **Hoechst 33342** (2 μ M) at 37 °C for 20 min. (c, d) Cells pretreated with 5 μ M and 80 μ M of 5-FU for 48 h and stained with **DDAB** and **Hoechst 33342**; (e) Cells pretreated with BNPP (100 μ M) and further stained with **DDAB** and Hoechst 33342. Scar bars= 20 μ m.

3.7. Visualization of CE2 levels in living mice and organs

The prominent features of **DDAB** encouraged us to further investigate the suitability of the probe for measuring CE2 in the context of living animals. To this end, Balb/c nu/nu mice were selected and administrated of **DDAB** (200 μ M) *via* intraperitoneal injection. After 30 min, the animals were observed by an *in vivo* fluorescence imaging system. Remarkable fluorescence images were acquired from inside the mouse body, especially in small intestine and liver (Fig. 5b, c). In sharp contrast, the mouse injected with PBS did not elicit any red fluorescence under the same imaging conditions (Fig. 5a). To confirm the tissue distribution of CE2 in mouse, one of the animals was dissected to isolate various tissues and further stained with **DDAB** (20 μ M). Interesting, strong fluorescence in the liver and small intestine was observed (Fig. 6A). The activities of CE2 in homogenates from various mouse tissues were also assayed by **DDAB**. The results revealed that the activities of CE2 in small intestine and liver were much higher than that in other organs, such as spleen, heart, and lung (Fig. 6B). Furthermore, these results agreed well with the protein expression of CE2 in these tissues, which were determined by an antibody-based method (Fig. 6B). All these findings revealed that CE2 were abundant in mouse small intestine and liver, and **DDAB** was capable of measuring endogenous CE2 in living animals and tissues.

Fig. 5 Representative fluorescence images of visualizing CE2 levels in nude mice using **DDAB**. (a) PBS-injected mouse; (b, c) **DDAB** (200 μ M, 150 μ L in PBS) injected mouse. Image was taken after injection for 30 min with an excitation laser at 630 nm and an emission filter of 700 ± 10 nm.

Fig. 6 (A) Fluorescence responses to *ex vivo*-dissected organs: (1) liver, (2) small intestine, (3) spleen, (4) lung, and (5) heart. Images were taken from 700 ± 10 nm fluorescence collection window. $\lambda_{\text{ex}} = 630$ nm. (B) Qualitative determination of CE2 level and activity in *ex vivo* dissected organs.

4. Conclusions

In summary, a novel near-infrared fluorescent probe **DDAB** for highly selective and sensitive detection of CE2 in living cells and *in vivo* has been developed for the first time. This ‘turn on’ NIR probe can selectively respond CE2 under physiological conditions, accompanied with sig-

nificant changes in both color and fluorescence spectrum. Cell experiments also demonstrate that **DDAB** can be used for fluorescence imaging in living cells with low cytotoxicity and satisfactory cell membrane permeability. Furthermore, **DDAB** has been successfully applied to visualize endogenous CE2 in various tissues and *in vivo*. All these features of **DDAB** suggest that this colorimetric NIR fluorescent probe holds great potential for further investigation on the biological functions of CE2 in complex systems.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at

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Table 1. Kinetic parameters for DDAB hydrolysis in different enzyme sources.

Enzyme sources	$V_{\max}$ (nmol/min/mg)	K_m (μ M)	CL_{int} (mL/min/mg)
HIM	8.88 $\pm$ 0.32	0.95 $\pm$ 0.13	9.35
HLM	16.5 $\pm$ 0.74	1.83 $\pm$ 0.24	9.02
HKM	9.38 $\pm$ 0.59	1.43 $\pm$ 0.14	6.56
CE2	20.40 $\pm$ 0.66	1.92 $\pm$ 0.31	10.63

Scheme 1 The structure of DDAB and its fluorescence response towards CE2.

Scheme 1

Fig. 1 Spectral changes of **DDAB** during its reaction with human CE2 in PBS (pH=7.4, 100 mM) at 37 °C. UV-vis absorption (A) and emission spectra changes (B) of **DDAB** (10 μ M) in the presence of CE2 (5 μ g/mL). λ_{ex} = 600 nm. Color and emission color (under a 365 nm light) changes are inserted, respectively.

Fig. 2 (A) Fluorescence responses of **DDAB** (10 μ M) towards various species of enzymes at 37 $^{\circ}$ C for 30 min. CE1 and CE2 (5 μ g/mL), carbonic anhydrase I (CA, 5 μ g/mL), α -chymotrypsin (α -CT, 5 μ g/mL), pepsin (5 μ g/mL), trypsin (5 μ g/mL), α 1-Acid Glycoprotein (AAG, 5 μ g/mL), bovine serum albumin (BSA, 50 μ g/mL), human serum albumin (HSA, 50 μ g/mL), acetylcholinesterase (AChE, 5 μ g/mL), butyrylcholinesterase (BChE, 20 U/L), paraoxonase (PON1 and PON2, 5 μ g/mL). (B) Inhibitory effects of specific inhibitors of human esterases on **DDAB** (10 μ M) hydrolysis in HLM (4 μ g/mL), HIM (4 μ g/mL) HKM (4 μ g/mL) and CE2 (2 μ g/mL). Data are shown as mean $\pm$ SD. The spectra were measured in phosphate buffer (PBS)-acetonitrile (v/v=1:1, pH 7.4). λ_{ex} = 600 nm.

Fig. 3 Confocal fluorescence images of SKOV-3 cells co-stained with **DDAB** and **Hoechst 33342**. (a-d) Cells treated with **DDAB** (20 μ M) and **Hoechst 33342** (2 μ M) at 37 $^{\circ}$ C for 20 min; (e-g) Cells pretreated with BNPP (100 μ M) and further stained with **DDAB** (20 μ M) and **Hoechst 33342** (2 μ M). (b, e) Fluorescence images from Hoechst 3342 (nuclear staining); (c, f) images from DDAB; (d, g) overlay of the blue and red channels. Scar bars= 30 μ m..

Fig. 4 Confocal fluorescence images of HL-7702 cells costained with **DDAB** and **Hoechst 33342** (nuclear staining). (a) Cells treated with Hoechst 3342 (2 μ M)), (b-e)

Cells treated with **DDAB** (20 μ M) and **Hoechst 33342** (2 μ M) at 37 $^{\circ}$ C for 20 min. (c, d) Cells pretreated with 5 μ M and 80 μ M of 5-FU for 48 h and stained with **DDAB** and **Hoechst 33342**; (e) Cells pretreated with BNPP (100 μ M) and further stained with **DDAB** and Hoechst 3342. Scar bars= 20 μ m.

Fig. 5 Representative fluorescence images of visualizing CE2 levels in nude mice using **DDAB**. (a) PBS-injected mouse; (b, c) **DDAB** (200 μ M, 150 μ L in PBS) injected mouse. Image was taken after injection for 30 min with an excitation laser at 630 nm and an emission filter of 700 ± 10 nm.

Fig. 6 (A) Fluorescence responses to *ex vivo*-dissected organs: (1) liver, (2) small intestine, (3) spleen, (4) lung, and (5) heart. Images were taken from 700 ± 10 nm fluorescence collection window. $\lambda_{\text{ex}} = 630$ nm. (B) Qualitative determination of CE2 level and activity in *ex vivo* dissected organs.

Graphical abstract

A colorimetric near-infrared fluorescent probe (**DDAB**) for highly selective and sensitive detection of carboxylesterase 2 (CE2) has been designed and well-characterized. This newly developed probe can be used for sensing CE2 in living cells and animals, and holds great promise for exploring the biological functions of CE2 in complex biological systems.