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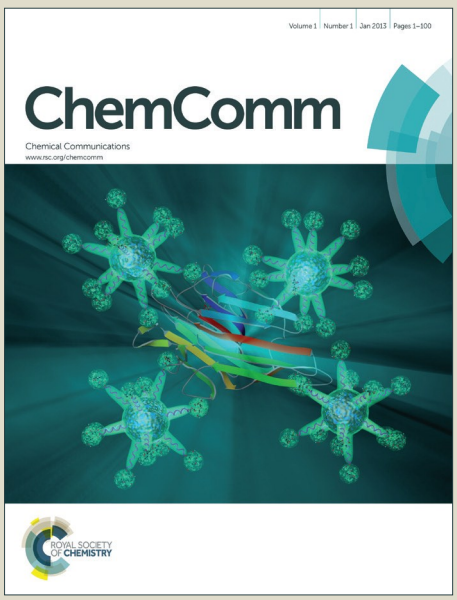

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A bioluminescent sensor for highly selective and sensitive detection of human carboxylesterase 1 in complex biological samples†

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A highly selective and sensitive bioluminescent sensor (DME) for human carboxylesterase 1 (hCE1) has been developed and well characterized. DME could be used for real-time monitoring of hCE1 activities in complex biological samples and for bio-imaging of endogenous hCE1 in living cells.

Carboxylesterases (CEs) are members of the serine esterase superfamily that are localized in various mammalian cells. Human carboxylesterase 1 (hCE1), one of the most abundant CEs in human, responsible for hydrolysis of numerous ester- or amide-containing compounds.[1] Compared with other CEs, hCE1 predominantly exists in liver tissue and prefers to hydrolyze substrate with a small alcohol group and large acyl group.[2] In human body, hCE1 plays a pivotal role in a wide range of physiological processes and cellular functions such as fatty acid metabolism and cholesterol hydrolysis, while dysfunction of hCE1 is known to be related to atherosclerosis and hypercholesterolemia.[3] Moreover, as one of the most important drug metabolizing enzymes, hCE1 is responsible for metabolism and detoxication of ester-containing compounds. The distribution and function of hCE1 will affect the clinical efficacy and outcomes of various hCE1-substrate drugs.[4] However, many factors such as genetic polymorphisms, age, and disease status, have been proved to cause large differences in both expression and function of hCE1 among various tissues or individuals.[5] Hence, it is necessary to develop highly selective and practical methods for precise monitoring of hCE1 activities in complex biological samples, which will be very helpful for drug discovery and clinical practice and also facilitate the further studies on the lipid metabolism-disease pathogenesis.

To quantify hCE1 in biological samples, several strategies including qRT-PCR, western blotting and proteomic techniques have been developed. However, such methods only can evaluate mRNA or protein levels of hCE1 rather than its real function.[6] Notably, activity-based molecule probes for target enzyme(s) are highly valuable for biomedical researches, due to they can directly measure the activity of a given enzyme under mild and biological-relevant conditions. Recently, we reported a selectively fluorescent probe (BMBT) which could be used to quantify the hCE1 activities in liver microsomes.[7] However, BMBT-based assay suffers from a major drawback due to the short emission wavelength of the hydrolyzed product, the fluorescent signal of which could be interfered by autofluorescence of biological matrix. This defect of BMBT strongly limits its wide applications in complex biological samples containing trace amounts of hCE1.

To overcome strong background fluorescence signals and detect hCE1 activities in complex biological samples more accurately, the present study turn to bioluminescent sensor. The emit light of bioluminescence originates from the chemical reaction of bioluminescent enzymes (luciferases) catalysis its specific substrate (luciferin).[8] Compare to fluorescent probes, bioluminescent sensors do not require excitation light to produce emission light, thus bioluminescence-based analysis could strongly refrain from interferences by absorption of biological matrix, as well as avoid phototoxicity to intact cells upon excitation light. Furthermore, there are virtually no endogenous light-emitting phenomenon in human living cells and tissues. Hence, the background signal for bioluminescence can be negligible and such high signal-to-noise values are superior to fluorescence detection for monitoring of target analyte in complex biological samples. These advantages make bioluminescent sensor based assay as a powerful new modality for biological and biomedical researches. Unfortunately, to date, none of the bioluminescent sensors for hCE1 have been reported yet.

Among the reported bioluminescent system, firefly luciferin-luciferase reaction is the most important and widely used system. The free luciferin can be masked with distinctive substitutes, which is an ingenious strategy to design bioluminescent sensors for detecting enzymes activity or evaluating bioactive small molecules.[9] From the view of chemical structure, the native D-luciferin contains a large carboxyl group, its ester derivatives with a small alcohol group may serve as good substrates for hCE1. To validate this assumption, a bioluminescent sensor (**DME**) for hCE1 was designed and synthesized by introduction of a methanol moiety into D-luciferin. The detail synthetic procedure for **DME** are described in the Electronic Supplementary Information (Scheme S1, ESI†), while the chemical structure of **DME** is fully characterized by ^{1}H NMR and ^{13}C NMR spectra (Fig.S16-17, ESI†).

The principle of bioluminescence monitoring of hCE1 activity is

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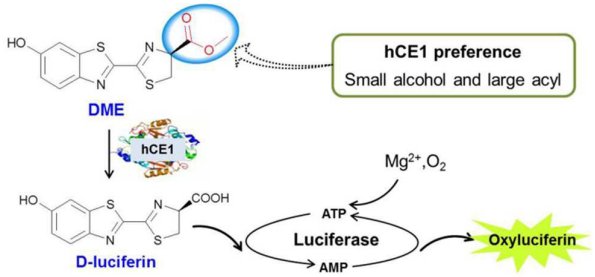

Scheme 1 The structure of **DME** and the proposed hydrolysis reaction for detection hCE1 activity using bioluminescence.

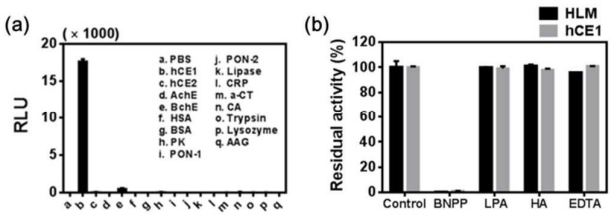

Fig. 1 (a) Luminescence responses of **DME** (3 μM) towards different species hydrolases. (b) The inhibitory effects of series esterase inhibitors on the hydrolysis of **DME** in HLM and hCE1. Data were presented as mean ± SEM from three repeat tests.

shown in Scheme 1. Upon addition of hCE1, **DME** could be rapidly converted to D-luciferin, which then initiated the firefly luciferase–luciferin system to generate bioluminescent signal. To ensure the applicability of this newly synthesized bioluminescent sensor, **DME** should not be a substrate of luciferase. As shown in Fig. S1 (ESI†), **DME** could not react with luciferase, due to no bioluminescent signal was detected following 30 min incubation with luciferin detection reagent (LDR). The effects of pH values on the enzymatic activity of hCE1 were also investigated. The results demonstrated that hCE1 exhibited the best catalytic efficacy towards **DME** hydrolysis in phosphate buffer (PBS) at pH 6-7(Fig. S2c, ESI†), which is consistent with former report that the pH 6.5 is optimal for hCE1-mediated hydrolysis.[10] Furthermore, luciferase could work well at pH 4.3-7.0 and **DME** was stable at this range of pH values, (Fig. S2a&2b, ESI†). Based on an integrative consideration, pH 6.5 was selected as the optimal pH value to maximize the luminescence signal for hCE1 detection. **DME** could be readily hydrolysed and formed a single metabolite upon addition of hCE1. The metabolite of **DME** was fully characterized as D-luciferin, by comparing the LC retention times, UV spectra, and MS/MS spectra of *in situ*-formed product with standard (Fig. S3, ESI†). These results demonstrated that **DME** could be used as a candidate sensor for measuring hCE1 activity under biological-relevant conditions.

To investigate whether **DME** could serve as an activity-based sensor for hCE1, the selectivity of **DME** hydrolysis was investigated using a panel of human hydrolases. As shown in Fig 1a, **DME** was found to show an excellent preference for hCE1, with eliciting a markedly increase bioluminescence intensity. In sharp contrary, other hydrolases including human carboxylesterase 2 (hCE2), cholinesterases (AChE and BChE), paraoxonases (PON1 and PON2), lipase, human serum albumin (HSA), bovine serum albumin (BSA), proteinase K (PK), c-reactionprotein (CRP), a-chymotrypsin (a-CT), carbonic anhydrase I (CA), trypsin, lysozyme, and a1-acid glycoprotein (AAG) led to negligible changes in bioluminescence intensities. To test the anti-interference ability of this probe reaction in

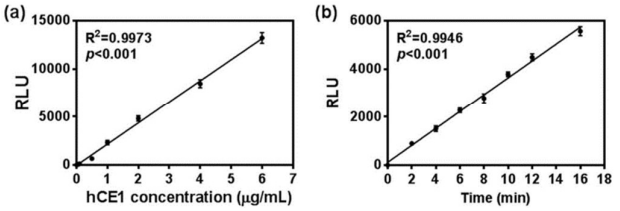

Fig. 2 Luminescence response of **DME** (3 μM) upon addition of increasing concentrations of hCE1 (0.01-12 μg·mL^{-1}) in PBS (pH 6.5) at 37 °C for 10 min.

complex biological system, the effects of various endogenous molecules (common amino acids and metal ions) on hCE1 activity detection were also determined. The unique bioluminescence performance of **DME** towards hCE1 was not influenced following co-incubation with common biological matrix (Fig. S4, ESI†). These results clearly indicated that **DME** was a highly selective substrate for hCE1.

To further validate the selectivity of **DME** towards hCE1 in complex biological samples, the inhibition assays of various selective esterase inhibitors (Table S1, ESI†) on **DME** hydrolysis were conducted in both hCE1 and pooled human liver microsomes (HLMs). As shown in Fig 1b, BNPP (a potent inhibitor of hCEs)[11] could completely inhibit the formation of D-luciferin in both hCE1 and HLMs, while the specific inhibitors of other human esterases including LPA (a selective inhibitor of hCE2), EDTA (a selective inhibitor of PONs), and HA (a selective inhibitor of AChE)[12] displayed negligible inhibitory effects toward **DME** hydrolysis. Encouraged by the above results, we then investigated the potential applications of **DME** for the high-throughput screening (HTS) of potential hCE1 inhibitors which might cause clinical drug-drug interactions. To demonstrate this potential use, a HTS test using **DME** hydrolysis as the probe reaction was performed. As shown in Fig. S5 (ESI†), the inhibitory tendency and the IC_{50} values of both BNPP and bavachinin (a naturally occurring hCE1 inhibitor) in recombinant hCE1 and HLMs were much closed. These results suggested that the **DME**-based assay could serve as a promising way for HTS of potential hCE1 modulators using tissue preparations as enzyme sources.

We next sought to determine whether **DME** was sensitive enough to detect the real activities of hCE1 in complex biological samples. To this end, the linear luminescence responses with enzyme concentrations were investigated firstly. The luminescence intensity enhanced gradually with the increasing hCE1 concentrations, while an excellent linear correlation (R^2=0.9973, p<0.001) between the luminescence responses and hCE1 concentrations was observed (Fig. 2a). In addition, a time course study on **DME** hydrolysis was conducted and the results showed that the luminescence responses were linearly related to the incubation times up to 16 minutes (Fig. 2b). Therefore, further enzymatic kinetic analysis and quantitative determinations for hCE1-mediated **DME** hydrolysis were conducted within 16 min. The limit of detection (3σ/slope) of **DME** for hCE1 detection was also determined as low as 0.01 μg·mL^{-1}, which was 20-fold more sensitive than the previously reported fluorescent substrate BMBT-based method. Such high sensitivity could be partially attributed to the very low background auto-luminescence signal from biological matrix and the rapid hydrolysis of **DME** upon addition of hCE1.

The kinetic behaviour of probe substrate to target enzyme

Table 1 Kinetic parameters for **DME** hydrolysis in different enzyme sources.

Enzyme sources	V_{max} (μmol·min^{-1}·mg^{-1})	K_m (μM)	CL_{int} (L·min^{-1}·mg^{-1})
HLM	1441±69	3.60±0.65	400.27
hCE1	1208±38	4.51±0.50	268.44

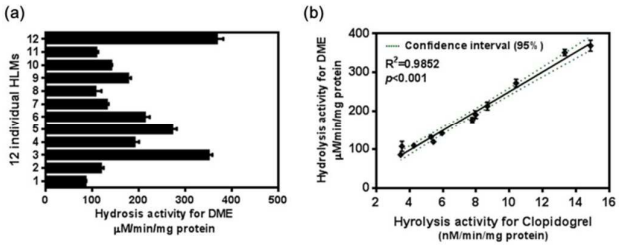

Fig. 3 (a) The hydrolytic activities of **DME** in 12 individual HLMs. (b) Correlation analysis between the hydrolytic rates of clopidogrel and the hydrolytic rates of **DME** in these 12 individual HLMs (n = 12).

was very important for the quantitative applications of activity-based probes.[13] To characterize the enzymatic kinetics of hCE1-mediated **DME** hydrolysis, two different enzyme sources including recombinant hCE1 and HLMs were used. In both hCE1 and HLMs incubation systems, **DME** hydrolysis followed the classic Michaelis-Menten kinetics, evidenced by the corresponding Eadie-Hofstee plots (Fig. S6, ESI†). Furthermore, **DME** hydrolysis in human liver preparations displayed closed K_m values to that in hCE1, implying that hCE1 was the predominant enzyme responsible for **DME** hydrolysis in human liver preparations. The kinetic parameters also demonstrated that **DME** hydrolysis in both hCE1 and HLM showed high affinity (K_m < 5 μM) and excellent reactivity (CL_{int} > 200 L·min^{-1}·mg^{-1} protein) (Table 1). These results clearly demonstrated that **DME** hydrolysis exhibited excellent selectivity, good reactivity and ideal kinetic behaviour, which prompted us to apply this sensor for quantitative measurement hCE1 activities in various biological samples.

To assess the applicability of this newly developed hCE1 sensor, **DME** was used to determine the real activities of hCE1 in various biological systems including human tissue and cell preparations. As depicted in Fig. 3a, 12 HLM samples from individual donors displayed varied enzymatic activities toward **DME**, in which about 4.3-fold differences in hCE1 activities was observed. This finding agreed well with the previous literatures reported differences in enzymatic activity measured by other hCE1 substrates.[14] Furthermore, an excellent linear correlation (R^2=0.9626, P<0.001) was presented between **DME** hydrolysis and clopidogrel (a known specific substrate of hCE1) hydrolysis in these individual HLM samples.[15] Next, the **DME**-based assay was further applied to measure hCE1 activities in microsomes from different tissues. These tissue preparations showed extremely different hCE1 activities, while HLMs showed the highest hCE1 activity. These results were further validated by western blot (Fig. 4a), and a good relevance was observed between the rate of **DME** hydrolysis and hCE1 protein levels among various human tissues (Fig. 4b). Furthermore, **DME** was further applied to measure the hCE1 activities in cell homogenates from different human tumor cell lines. As shown in Fig. 4c and 4d, the hCE1 activities in different tumor cells agreed well with the relative abundance of hCE1 in both mRNA

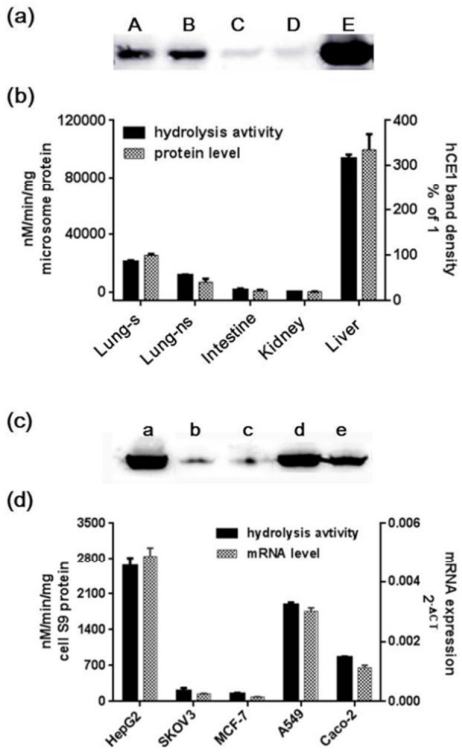

Fig. 4 (a) Western blot analysis of hCE1 expression in microsomes from different human tissues. A, lung microsomes (smoker); B, lung microsomes (non-smoker); C, kidney microsomes; D, intestinal microsomes; E, liver microsomes. (b) Comparison of the hydrolytic activities and expression levels of hCE1 in different tissue microsomes. (c) Western blot analysis for hCE1 protein expression of different tumor cells S9. a, HepG2 cell; b, SKOV3 cell; c, MCF-7 cell; d, A549 cell; e, Caco-2 cell; (d) The hydrolytic activities of **DME** (left) and mRNA expression (right) in different tumor cells S9 fraction.

(detected by real-time PCR) and protein expression (detected by western blot). In addition, **DME** hydrolysis in these tissue and cell preparations used above could be significantly inhibited by BNPP (a potent inhibitor of hCEs), while LPA (a selective inhibitor of hCE2) displayed negligible inhibitory effects toward this biotransformation (Fig.S7&S8, ESI†). All these results demonstrated that **DME** could be used to selectively and sensitively determine the real activities of hCE1 in complex biological samples and the quantification was highly reliable.

Finally, the potential applications of **DME** for the bio-imaging and real-time monitoring of endogenous hCE1 in living cells were further explored. Prior to cell experiment, the cytotoxicity of **DME** was firstly investigated by a standard MTT assay. As shown in Fig. S9 (ESI†), **DME** displayed extremely low cytotoxicity. SKOV-3-Luc$^+$ cells remained in good conditions following treatment with a high dosage of **DME** (150 μM) for 48 h. In these cases, **DME** (50 μM) was co-incubated with SKOV-3-Luc$^+$ cells and the bioluminescence images were conducted. As shown in Fig. 5, the bioluminescence intensity increased proportionally with increasing cell numbers. Furthermore, a strong correlation between cell numbers and light emission was observed under microplate reader measurement (Fig. S12b). After co-incubation with **DME**, time-course kinetics of the bioluminescence signal in SKOV-3-Luc$^+$ cells was also tested. The bioluminescence intensity gradually increased to a

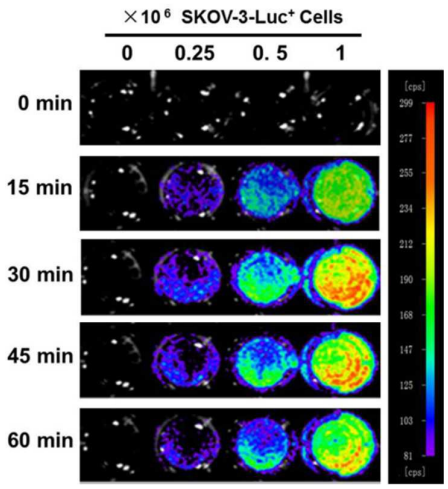

Fig. 5 Bio-imaging of bioluminescence signal in SKOV3 cells co-expressing luciferase. Cell concentrations ranging from 0.25 x 10^6 to 1 x 10^6 cells were plated in a 24-well plate and images were captured after addition of **DME** to the media. Wells containing medium only with or without **DME** served as negative controls.

maximum, then decayed over time (Fig. 5). Time of maximum peak was different between **DME** and D-luciferin. Maximum bioluminescence intensity was appeared at 30 min following **DME** addition into the culture medium while 20 min for native D-luciferin (Fig. S11, Fig. S12a, ESI†). The delay of maximum peak time could be explained by that **DME** should be absorbed into the cells and then hydrolysed to form D-luciferin through endogenous hCE1. These results indicated that **DME** could be used to real-time monitor the functions of endogenous hCE1 and thus could be used for exploring hCE1 related biological processes in living cells.

In summary, this study describes the synthesis, characterization, and biological applications of a practical bioluminescent sensor for hCE1 for the first time. The newly developed activity-based probe displays excellent selectivity and sensitivity towards hCE1 over other human hydrolases and biologically relevant matrix. **DME** could be readily hydrolysed by hCE1 and release D-luciferin, a native substrate of firefly luciferase, allowing assay by bioluminescence system. **DME** could serve as a reliable probe to measure the real activities of hCE1 in complex biological systems including tissue and cell preparations, which could be applied for high-throughput screening and characterization of hCE1 modulators using human tissue or cell preparations as enzyme sources rather than expensive recombinant hCE1. Furthermore, **DME** could be used to real-time monitor the function of endogenous hCE1 in living cells co-expressing luciferase. All these findings suggested that **DME** could serve as a highly practical bioluminescent sensor for monitoring hCE1 activities in complex biological systems and for exploring the biological functions and medicinal roles of hCE1 in living systems.

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