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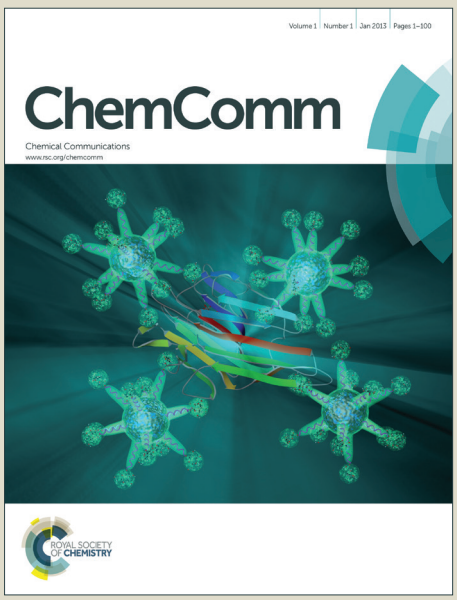

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A highly selective long-wavelength fluorescent probe for human carboxylesterase 2 and its biomedical applications†

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A highly selective long-wavelength fluorescent probe TCFB has been developed for the detection of hCE2. The probe can be used for real-time monitoring of hCE2 activity in complex biological systems.

Carboxylesterases (CEs) are members of the serine hydrolase superfamily found in numerous animal species.[1] CEs catalyze hydrolytic and transesterification reactions with a vast number of endogenous and exogenous substrates including fatty acid esters, environmental toxins and ester-containing drugs.[2] In human, the majority of CEs belong to the CE1 and CE2 gene families, while human carboxylesterase 1 (hCE1) and carboxylesterase 2 (hCE2), have been identified as the major CEs distributed in human tissues.[3] hCE1 and hCE2 are differentiated on the basis of tissue distribution and substrate specificity. As the major CE isoform distributed in human intestine and tumour tissues, hCE2 plays important role in oral bioavailability of prodrugs, and the treatment outcomes of ester anti-cancer agents.[4] Notably, it has been reported that the individual difference in hCE2 activity are closely associated with the clinical outcome and toxicity of irinotecan, a widely used anticancer agent derived from camptothecin.[5]

To date, several methods including immunological method and mass spectrometry-based proteomic technique, have been developed for hCE2 detection and quantification.[3b,6] The immunological technique is relatively labour-intensive and the associated reagents are relatively high cost. The mass spectrometry-based technique is relatively complicated, and the operation is limited by some stringent requirements (such as expensive instruments and high qualified operators). Moreover, these methods can only evaluate the protein level rather than its activity. Thus, it is highly desired to develop a practical, sensitive and selective method for the precise measurement of the real activity of hCE2 in biological samples. Compared with antibody and proteomic-based techniques, activity-based probes including non-fluorescent and fluorescent probes can selectively and directly measure the enzymatic activity of hCE2.[7] Among the reported selective probes of human enzymes, fluorescent probes have attracted increasing attention because of their inherent advantages, such as highly sensitive, non-destructive, easily-conducted, as well as applicable to high-throughput screening.[8]

In recent years, a few fluorescent probes including fluorescein diacetate (FD), have been developed to detect hCE2 activity.[7c] However, the emission wavelengths of these fluoresce probes are under 600 nm, which blocked their widely applications in bio-imaging or other uses in complex biological systems, due to the interferences by absorption and autofluorescence of biological matrix. It is well known that fluorescent probes with emission in long-wavelength region are more suitable for practical measurements and biological imaging.[9] Thus, a highly selective long-wavelength fluorescent probe for hCE2 is urgently desirable.

Among the reported long-wavelength fluorophores, 2-dicyanomethylene-3-cyano-4, 5, 5-trimethyl-2, 5-dihydrofuran is preferred for bio-imaging of some bio-species, by virtue of its strong electron-withdrawing properties induced by the conjugated system and three cyano groups.[10] Herein, a long-wavelength fluorescent probe **TCFB** was designed for sensing of hCE2 activity, based on its substrate preference and the principle of the intramolecular charge transfer (ICT).[11] **TCFB** can be readily synthesized by using 1-(3-cyano-2-dicyanomethylen-5, 5-dimethyl-2, 5-dihydrofuran-4-yl)-2-(4-hydroxylphenyl) ethane (**TCF**) and benzoyl chloride as the starting materials (**Scheme S1, ESI**†) and its structure was fully confirmed by ^{1}H-NMR, ^{13}C-NMR and HRMS spectra (**Fig. S1-S3, ESI**†). The ICT progress can be completely blocked by introducing benzoyloxy group as a quenching and reacting moiety to **TCF**.

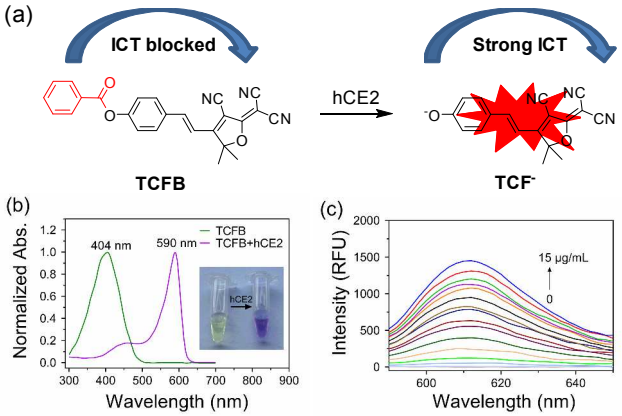

Fig. 1 (a) The structure of **TCFB** and its fluorescence response toward hCE2. (b) Normalized absorption spectra of **TCFB** (20 μM) upon addition of hCE2 (15 μg/mL). (c) Fluorescence response of **TCFB** (20 μM) in the presence of increasing concentrations of hCE2. The spectra were measured in PBS-acetonitrile (V: V=1:1, pH 7.4) at 37 °C for 30 min. λ_{ex}=560 nm.

First, the absorption and emission properties of **TCFB** toward hCE2 in PBS-acetonitrile (V: V=1:1, pH 7.4) were investigated. As shown in **Fig. 1b, TCFB** exhibited an absorption band at 404 nm in buffer without hCE2. Upon addition of hCE2, the absorption at 404 nm decreased evidently, while a new absorption band at 590 nm emerged. Such a large red shift (186 nm) in the absorption behaviour changed the colour of the solution from yellow to bluish violet, indicating that **TCFB** can serve as a ''naked-eye'' colorimetric indicator for hCE2. Consistently, co-incubation of **TCFB** with hCE2 led to the occurrence of a fluorescence emission band with a maximum value at 612 nm (upon excitation at 560 nm) (**Fig. 1c**). The in situ-formed product was identified as **TCF**, by comparison of the LC retention times, UV and MS spectra with standard (**Fig. S4, ESI†**). The suitable pH range for hCE2 detection by **TCFB** was then tested. It was found that the fluorescence intensity of **TCFB** was quite stable at pH 2–12 (**Fig. S5, ESI†**), while the fluorescence intensity of **TCF** (Φ_{fl}=0.0022) could be enhanced remarkably in the neutral pH range from 5.3 to 6.8, and then reach a plateau at pH 6.8–12. These results demonstrated that **TCFB** could function properly at physiological conditions (pH 7.4). Time course studies (**Fig. S6, ESI†**) showed that the fluorescence intensity was linearly related to incubation time within 30 min. Therefore, the further enzyme kinetic analysis was conducted within 30 min.

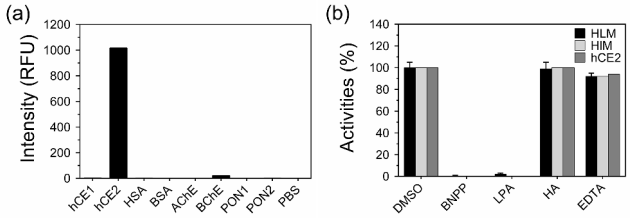

Fig. 2 (a) Fluorescence responses of **TCFB** (20 μM) towards various species of enzymes. (b) Inhibitory effects of specific inhibitors of human esterases on **TCFB** (20 μM) hydrolysis in HLM (20 μg/mL), HIM (20 μg/mL) and hCE2 (10 μg/mL). The same amount of DMSO (2%, V/V) without any inhibitor was used as blank control. Data are shown as mean ± SEM. The spectra were measured in PBS-acetonitrile (V: V=1:1, pH 7.4) at 37 °C for 30 min. λ_{ex}=560 nm.

Next, the selectivity of **TCFB** hydrolysis was investigated. As shown in **Fig 2a**, hCE2 elicited a dramatic increase in the fluorescence intensity at 612 nm, while other hydrolytic enzymes including hCE1, cholinesterases (AChE and BChE), paraoxonases (PON1 and PON2), human serum albumin (HSA), and bovine serum albumin (BSA) led to negligible changes in fluorescence intensities. To further investigate the selectivity of **TCFB** hydrolysis in complex biological systems, the inhibition assays were conducted in human tissue preparations by using a series of selective esterase inhibitors. As shown in **Fig 2b**, BNPP (a potent inhibitor of hCEs)[12] and LPA (a selective inhibitor of hCE2) can completely inhibit **TCFB** hydrolysis in human liver microsomes (HLM) and human intestine microsomes (HIM),[7a] while inhibitors of other human esterases including EDTA (a selective inhibitor of PON)[13] and HA (a selective inhibitor of AChE)[14] did not inhibit this reaction. These results suggested that **TCFB** hydrolysis was selectively catalyzed by hCE2, and it could serve as an efficient tool for measuring the real activity of hCE2 in complex biological matrixes.

For accurate measurement of the real activity of target enzyme, the linear fluorescent response with enzyme concentration was very essential. Under the optimized conditions, the fluorescence intensity gradually enhanced with increasing hCE2 concentration, and a good linearity relationship (R^2>0.99) between the fluorescence intensity at 612 nm and hCE2 concentrations in the range of 0 to 15 μg/mL was obtained (**Fig. 3a**). The limit of detection (3σ/slope) of **TCFB** was also determined to be 0.46 μg/mL. Such high sensitivity could be attributed to the very low background fluorescence signal of **TCFB** around 612 nm caused by introducing benzoyloxy moiety, which completely blocked the ICT process.

The kinetic behaviour was also important for the quantitative applications of activity-based probes.[15] In this study, the enzymatic kinetics of **TCFB** hydrolysis was characterized in different enzyme sources including recombinant hCE2, HLM and HIM. **TCFB** hydrolysis in hCE2, HLM and HIM followed the typical Michaelis-Menten kinetics, which was evidenced by the corresponding Eadie-Hofstee plots (**Fig. S7, ESI†**). As shown in **Table S1**, **TCFB** hydrolysis in human tissue preparations displayed very similar K_m values as it in hCE2, implying that hCE2 was the predominant enzyme responsible for **TCFB** hydrolysis in human tissues. Furthermore, **TCFB** hydrolysis in human tissue preparations displayed good reactivity (k_{cat}/K_m >5 mL/min/mg protein). These findings demonstrated that hCE2-mediated **TCFB** hydrolysis exhibited excellent selectivity, ideal kinetic behaviour and good reactivity, which inspired us to use this probe reaction for potential applications in biological samples containing multiple enzymes.

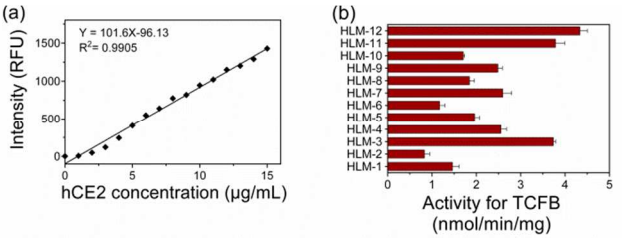

Fig. 3 (a) The fluorescence intensity at 612 nm of **TCFB** (20 μM) was linearly related to the concentration of hCE2 (0–15 μg/mL). (b) The catalytic activities of hCE2 in twelve individual HLMs by using **TCFB** as the probe substrate. The spectra were measured in PBS-acetonitrile (V: V=1:1, pH 7.4) at 37 °C for 30 min. λ_{ex}=560 nm.

Fig. 3b displayed the activities of **TCFB** hydrolysis in a panel of twelve individual HLM samples. As expected, the formation rates of **TCF** are highly consistent with the hydrolytic rates of FD (R^2=0.9204, P<0.0001), the commercial available probe of hCE2 (**Fig. S8, ESI†**). Furthermore, the inhibition profiles and the inhibition capability of LPA in HLM and HIM were also depicted, which were very similar to those in hCE2 (**Fig. S9, ESI†**). All of these findings strongly suggested that **TCFB** can be used as a highly selective florescence probe for rapid measuring the real activity of hCE2 in complex biological samples, as well as for high throughput screening of hCE2 modulators by using human tissue preparations as enzyme sources.

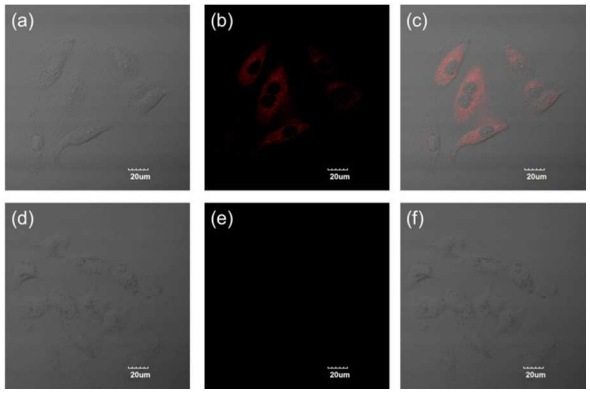

Fig. 4 Confocal fluorescence images of A549 cells. Cells incubated with **TCFB** (50 μM) for 30 min (top); cells pre-treated with LPA (100 μM) and then incubated with **TCFB** for 30 min (bottom). (a), (d) bright-field images; (b), (e) red emission (627 ± 50 nm); (c), (f) merged images. λ_{ex}=543 nm. Scale bars denote 20 μm.

We next explored the potential utility of **TCFB** for biological imaging of endogenous hCE2 in living A549 cells. The SRB assay showed that **TCFB** did not exhibit cytotoxicity at 200 μM under 37 °C for 4 h (**Fig. S10, ESI**†). It could be seen that A549 cells loaded with **TCFB** (50 μM) for 30 min at 37 °C showed an obvious red fluorescence (**Fig. 4b**), suggesting that **TCFB** was cell membrane permeable and could be efficiently hydrolyzed by intracellular hCE2. In sharp contrast, cells pre-treated with hCE2 selective inhibitor LPA (100 μM) did not give remarkable fluorescence enhancement (**Fig. 4e**). These results indicated that **TCFB** could be used for measuring the functions of hCE2 and exploring its related biological processes in living cells.

Additionally, docking and molecular dynamics approaches were utilized to provide deep insight into hCE2 mediated **TCFB** hydrolysis. As shown in **Fig. 5**, **TCFB** can be well docked into the catalytic site of hCE2, while the ester bond of **TCFB** was near to the catalytic amino acids in hCE2 (Ser-240, Glu-364, and His-478). As a result, the docking at the best binding mode gave rise to the lowest binding free energy (-9.45 kcal/mol). This was in agreement with our experimental result which demonstrated **TCFB** had high affinity for hCE2.[16] In sharp contrast, **TCFB** could not be docked into the catalytic site of hCE1, the binding site was on the surface of this protein (**Fig. S11, ESI**†). These findings indicated that **TCFB** was a good substrate of hCE2 but a poor substrate of hCE1.

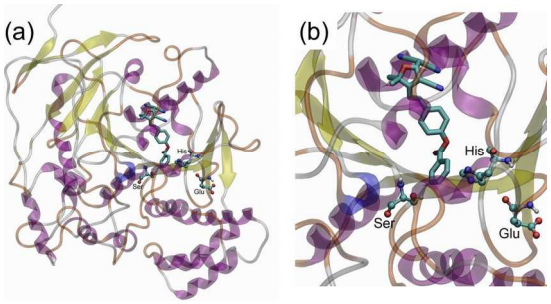

Fig. 5 The possible binding of the amino acids residues (Ser-240, Glu-364, and His-478) in active site triad of hCE2 with **TCFB**. (a) A stereo view of the crystal structure of modelling hCE2 and the stereodiagram of **TCFB** aligned in its active site. (b) Detail view of the binding site showed that the hydroxyl group of Ser-240 residue is close to the carbonyl carbon of **TCFB** and is consistent in both distance and orientation for nucleophilic attack.

In summary, an ICT-based fluorescent probe deriving from a long-wavelength emission fluorophore **TCF** has been developed for highly selective detection of hCE2. The probe can be efficiently hydrolyzed by hCE2 and release **TCF** which leads to a significant colour change and a remarkable long-wavelength emission in fluorescence spectrum, allowing the naked-eye visible and fluorescence analysis. **TCFB** hydrolysis displays excellent selectivity, high sensitivity, and ideal kinetic behaviour in human biological samples. Furthermore, **TCFB** has been successfully applied for real-time monitoring the real activities of hCE2 in complex biological samples even with hCE2 inhibitors, and bio-imaging of endogenous hCE2 in living cells. All of these findings suggest that **TCFB** can serve as a promising tool for exploring the real functions of hCE2 in complex biological systems.

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Notes and references

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