**Supplementary Information**

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

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Materials
Bis-p-nitrophenyl phosphate (BNPP) and loperamide (LPA) were obtained from TCI (Tokyo, Japan). Huperzine A (HA) and ethylene diamine tetraacetic acid (EDTA) were purchased from J&K Chemical (Beijing, China). Luciferin detection reagent (LDR) was obtained from Promega Biotech (Madison, USA). Recombinant human cholinesterase (AChE and BChE), a-chymotrypsin (a-CT, C4129), carboxic Anhydrase I (CA, C4396), trypsin, proteinase k (P2308), a1-Acid Glycoprotein (AAG, G9885), and human serum albumin (HSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Lysozyme (306A0416) was got from Solarbio (Beijing, China). Human paraoxonases 1 (PON1, v13111203) and human paraoxonases 2 (PON2, v13111202) were purchased from Bioworld Technology (USA). Recombinant expressed human carboxylesterases 1 (hCE1) and human carboxylesterases 2 (hCE2) were purchased from BD Biosciences (MA, USA). Pooled human liver microsomes (HLMs) and a panel of HLMs from twelve individuals were purchased from RILD (Shanghai, China). RNAiso Plus reagent, RNA PCR kit, and SYBR® Premix Ex Taq™ II Kit were purchased from Takara (Dalian, China). BCA protein assay kit, PVDF membrane, and Super ECL Plus were obtained from Beyotime (Beijing, China). Rabbit anti-human hCE1 antibody and HRP-conjugated goat anti-rabbit IgG were purchased from Abcam (UK). All other LC grade solvents were purchased from Tedia (USA). A stock solution of DME (30 mM) was prepared in DMSO and stored at -80 °C for future using.

Instruments
1H NMR and 13C NMR spectra were measured using a 700 MHz AVAVCE III HD 700MHz spectrometer. Luminescence measurements were conducted on Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek). The supernatants of reaction mixture were determined using a Shimadzu UFLC system coupled with a diode array detector (DAD) and a mass spectrometer (Shimadzu, Japan). Real time RT-PCR was conducted on Applied Biosystems StepOne™ Real-Time PCR System (Applied Biosystems, Foster City, CA). Immunoreactive bands on the PVDF membrane were scanned and evaluated using Fusion Fx5 820.WL.

Synthesis and structural characterization of DME
Under Ar atmosphere, pyridine hydrochloride (1155 mg, 10 mmol) was added to a solution of compound 1 (190 mg, 1 mmol) in sulfolane (2.5 mL). The reaction mixture was stirred at 180 °C for 6.0 h. After cooling to room temperature, the remaining residue was suspended in ethyl acetate (100 mL), then washed with H₂O (50 mL×2), brine (30 mL×1) and dried (Na₂SO₄). After evaporation of the solvent, the crude residue was purified by column chromatography on silica gel (petroleum ether/ethyl acetate = 3/1) to give compound 2 (162 mg, 92%) as a yellowish solid. \(^1\)H NMR (700 MHz, DMSO) δ 10.54 (s, 1H), 8.07 (d, \(J = 9.0\) Hz, 1H), 7.59 (d, \(J = 2.3\) Hz, 1H), 7.19 (dd, \(J = 9.0, 2.4\) Hz, 1H). \(^{13}\)C NMR (176 MHz, DMSO) δ 159.1, 145.8, 138.1, 132.8, 125.9, 119.1, 114.2, 107.1.

Thionyl chloride (238 mg, 2 mmol) was added to methanol (5 mL) at 0 °C. The resulting solution was stirred at 0 °C for 0.5 h, and then a solution of (S)-Cysteine 3 (62 mg, 0.5 mmol) in methanol (2.5 mL) was added. The reaction mixture was heated to reflux for 4.0 h and then concentrated to give compound 4 (88 mg, quantitative) as a yellow oil. The crude product obtained was used for subsequent reaction without further purification.

Under Ar atmosphere, compound 2 (79 mg, 0.45 mmol) and compound 4 (88 mg, 0.5 mmol) were suspended in MeCN (5 mL). A solution of K₂CO₃ (69 mg, 0.5 mmol) in 400 µL of H₂O was added to the mixture. The reaction mixture was stirred vigorously at room temperature for 2.0 h. Water (50 mL) was added and the pH was adjusted to 5-6 by the addition of a 1 M HCl solution. The mixture was extracted with ethyl acetate (50 mL×2) and the combined organic layers were washed with brine (60 mL×1) and dried (Na₂SO₄). After evaporation of the solvent, the crude residue was purified by column chromatography on silica gel (petroleum ether/ethyl acetate = 3/1) to give DME (83 mg, 63%) as a white solid. \(^1\)H NMR (700 MHz, DMSO) δ 10.26 (s, 1H), 7.98 (d, \(J = 8.9\) Hz, 1H), 7.46 (d, \(J = 2.2\) Hz, 1H), 7.08 (dd, \(J = 8.9, 2.3\) Hz, 1H), 5.53 (t, \(J = 9.1\) Hz, 1H), 3.81 (t, \(J = 11.2\) Hz, 1H), 3.77 (s, 3H), 3.70 (dd, \(J = 11.1, 8.5\) Hz, 1H). \(^{13}\)C NMR (176 MHz, DMSO) δ 170.8, 165.6, 157.9, 157.0, 146.7, 137.7, 125.4, 117.7, 107.3, 78.2, 53.0, 35.1.

**General procedure for measuring of carboxylesterase activities**

The incubation mixtures included a total volume of 50 µL 100 mM potassium phosphate buffer (pH=6.5) and human liver microsomes (HLM) or other mentioned esterase sources.
After pre-incubation at 37°C for 10 min, the reaction was initiated by the addition of the stock solution of \textbf{DME} (3 \, \mu M, final concentration). In all experiments, \textbf{DME} (30 mM in DMSO) was serially diluted to designed concentrations and the final concentrations of DMSO were below 1\% (v/v). For LC analysis, the reactions were terminated by adding 50 \, \mu L acetonitrile after 10 min incubation at 37 ºC, followed by centrifugation at 20,000\times g for 20 min by using Allegra 64R centrifuge, the aliquots of supernatant were then taken for LC-FD analysis. While for luminescence measurements, 50 \, \mu L of luciferin detection reagent (LDR) was added to the reaction mixture after 10 min incubation. And after incubate at room temperature for 20 min (stabilize the luminescent signal), the mixture were taken for further analysis. Control incubations without enzyme sources were conducted to confirm that metabolites formation was enzyme dependent.

\textbf{LC-DAD-ESI-MS analysis}

To characterize \textbf{DME} and its hydrolytic metabolite in hCE1, LC-DAD-ESI-MS was used. The supernatants of reaction mixture were assayed using Shimadzu UFLC system equipped with DAD and mass spectrometer. The chromatographic separation was achieved using a Shim-pack XR-ODS analytical column (150 mm \times 2.0 mm, 2 \, \mu m). The mobile phase consisted of CH$_3$CN (A) and water containing 0.2\% formic acid (B) with the following gradient: 0–2 min, 90-70 \% B; 2–8 min, 70-5 \% B. The system was operated at a flow rate of 0.4 mL/min and column temperature was kept at a temperature of 40°C. The UV detection of \textbf{DME} and its hydrolytic metabolite was achieved at 254 nm. The mass spectrometer was operated under the negative ion monitoring mode from m/z 50 to 1000. The detector voltage was set at -1.55 kV for positive ion detection. The curved desolvation line temperature (CDL) and the block heater temperature were both set at 250 °C. Other MS detection conditions were as follows: interface voltage, 4 kV; CDL voltage, 40 V; nebulizing gas (N$_2$) flow was 1.5 L·min$^{-1}$ and the drying gas (N$_2$) pressure set at 0.06 MPa. Data processing was performed using the software LC-MS Solution version 3.41 (Shimadzu, Kyoto, Japan).

\textbf{Enzymatic reaction phenotyping assays}

To evaluate the selectivity of \textbf{DME} towards hCE1, a series of enzymes with hydrolytic activity were investigated. Carboxylesterase (hCE1 and hCE2, 10 \, \mu g·mL$^{-1}$), cholinesterases
(AChE and BChE, 10 μg·mL⁻¹), human serum albumin (HSA, 2 μg·mL⁻¹), bovine serum albumin (BSA, 10 μg·mL⁻¹), proteinase K (10 μg·mL⁻¹), paraoxonases (PON1 and PON2, 10 μg/mL), lipase (10 μg·mL⁻¹), c-reaction protein (CRP, 10 μg·mL⁻¹), α-chymotrypsin (α-CT, 10 μg·mL⁻¹), carbonic anhydrase I (CA, 10 μg·mL⁻¹), trypsin (10 μg·mL⁻¹), lysozyme (10 μg·mL⁻¹), and α1-Acid Glycoprotein (AAG, 10 μg·mL⁻¹) were co-incubation with DME at 37 °C for 30 min. Luminescence were measured as described above.

**Chemical inhibition assays**

To verify DME hydrolysis was selectively mediated by hCE1, the inhibitory effects of different esterase inhibitors (Table 1) on D-fluorescein production were investigated using different enzyme resource. Briefly, each of the selective esterase inhibitors (100 µM) was pre-incubated with HLM (10 μg·mL⁻¹) or hCE1 (10 μg·mL⁻¹) at 37 °C for 10 min. Then reactions were initiated by adding DME (3 µM) into the mixtures. After the enzyme mixtures incubation at 37 °C for 10 min, luminescence measurements were conducted as described above. The residual enzyme activity (%) was determined by the percent of D-fluorescein production in the presence of known selective esterase inhibitors to the control (in the absence of inhibitors). To evaluate the inhibitory parameters, various concentrations of BNPP were added to reaction mixtures containing enzyme resources (HLM or hCE1) and DME. The half inhibition concentration (IC₅₀) was calculated by fitting to log (Con.) vs. residual enzyme activity (%) using GraphPad Prism 6.0.

**Enzyme kinetics analysis**

The Enzyme kinetics parameters were determined by incubating DME (2-60 µM) with HLM (10 μg·mL⁻¹) or hCE2 (10 μg·mL⁻¹) in 50 μL of 100 mM phosphate buffer solution (pH 6.5), respectively. Serial concentrations of DME (diluted with PBS) were incubated at 37 °C for 10 min. Then the reactions were initiated by adding HLM or hCE2 into the pre-incubated enzyme mixtures. After 10 min incubation, the formation of D-fluorescein was determined by measuring the luminescence intensity as described above. Kinetic parameters (Kₘ and Vₘₐₓ) were calculated by nonlinear regression analysis using the Michaelis-Menten plot in the GraphPad Prism 6.0 software.
Measurement of hCE1 in human liver microsomes and correlation studies

Based on the general procedure for assay carboxylesterase activity mentioned above, DME (3 μM) was incubated with a panel of 12 individual human liver microsomes (HLMs) at 37 °C. Protein concentrations of HLMs were optimized within a linear range response. The production of metabolite was measured using Synergy H1 Hybrid Multi-Mode Microplate Reader. Clopidogrel, a reported probe for hCE1, was used to assay hCE1 of the 12 individual HLMs as described before. The rates of DME hydrolysis in 12 individual HLMs were compared with the rates of clopidogrel hydrolysis to confirm the reliability for measuring hCE1 in HLMs using DME in HLM samples. The correlation between DME and BMBT hydrolysis was expressed by the linear regression coefficient (R²). P<0.005 was considered statistically significant.

Real-time RT-PCR analysis of hCE1 mRNA expression

The hCE1 expression in different tumor cell lines was determined with real-time RT-PCR analysis. Briefly, cells were cultured in 12-well plates and collected by RNAiso Plus reagent when they reached 90 % confluence. Then the total cellular RNA was extracted and reverse transcription was conducted for synthesis of cDNA using RNA PCR kit. Subsequently, real-time quantitative PCR was carried out utilized SYBR®Premix Ex Taq™ II Kit. The relative expression level for hCE1 was normalized by the C_T value of the human housekeeping gene GAPDH (2^-ΔCT formula). Data was analyzed by Applied Biosystems StepOne™ Real-Time PCR System software version 2.0.

Preparation of cell homogenates

For preparation of cell homogenates, cells were cultured in 75 cm² flasks and collected by trypsin when they reached 90% confluence. Then cells were pelleted and washed with ice-cold PBS to remove FBS. Ice-PBS was added to cell pellets and the cell suspension sonicated three times for 3 s (200 W) with at least 1 min on ice between two pulses. The cell lysate was centrifuged at 9000 g for 20 min and the supernatant (S9 fraction) was collected and kept at -80°C until use.

Western blot for hCE1 protein expression analysis
The protein concentrations of different tissue microsomes and different tumor cell lines were measured using BCA protein assay. Then 20 µg microsome proteins or 30 µg cell S9 proteins were electrophoretically separated on a 10% SDS-polyacrylamide gel and transferred to a PVDF membrane. The membrane was then probed with specific Rabbit anti-human hCE1 antibody. The second antibodies used goat anti-rabbit IgG antibody. Chemiluminescence was developed using the reagent Super ECL Plus. Immunoreactive bands on the PVDF membrane were scanned and evaluated using FusionCapt Advance.

**Cytotoxicity assays**

The cytotoxic effects of DME on SKOV-3 cells were evaluated by MTT assay as described by Mossmann. Briefly, cells (5×10^4 /mL, 200 µL) were seeded in 96-well plate in the Coy’5A culture medium containing 10% fetal bovine serum and maintained at 37 °C in a 5% CO₂ incubator for 24 h. Then, the cells were incubated with different concentrations of DME dissolved in DMSO (final concentration less than 0.5%) for another 48 h. Subsequently, MTT (5 µg·mL⁻¹) was added to each well keeping the final MTT concentration at 0.5 µg·mL⁻¹. After 4 h incubation, the medium was discarded and 0.15 ml DMSO was added to each well. Then the culture plates were shaken for 10 mins on an oscillator. The absorbance at 490 nm was measured with a Hybrid multi-mode microplate reader. Each sample was analyzed in triplicate.

**Bioluminescence imaging in living SKOV3 cells**

SKOV3-Luc+ cells were cultured in Coy’5A (10% FBS) at 37 °C in a 5% CO₂ incubator. For bioluminescence imaging, serial dilutions of SKOV3-Luc+ cells (0.25× 10^6 to 1 × 10^6/ml, 1 ml) were plated in 24-well cell culture plates. DME (50 µM) was added immediately before bioluminescence imaging. After that, this bioluminescence imaging were taken by using a NightOWL II LB 983 small animal in vivo imaging system containing a sensitive Charge Coupled Device (CCD) camera, with an emission filter of 600 ± 10 nm. For the assays with inhibitor, SKOV3-Luc+ cells (0.5 × 10^6/ml, 1 ml) were plated in 24-well cell culture plates and pretreated with BNPP (100 µM) or DMSO (V/V, 1%) for 20 min. DME (50 µM) was added immediately before bioluminescence imaging.
Kinetics of the bioluminescence signal analysis in living SKOV3 cells

SKOV3-Luc+ cells were cultured in Coy’5A (10% FBS) at 37 °C in a 5% CO$_2$ incubator. For Kinetics of the bioluminescence signal analysis, serial dilutions of SKOV3-Luc+ cells (1 to $10 \times 10^5$ cells/well) were added in 96-well white plates. DME (3 μM) was added immediately before measurement. The time course of bioluminescence intensity was measured using a microplate reader with 1 s integration at each time point. Changes in bioluminescence intensity over time were measured and are presented as total bioluminescence intensity value for each well.

Table S1 List of selective inhibitors of various human esterases

<table>
<thead>
<tr>
<th>Inhibitor name</th>
<th>Target enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>BNPP</td>
<td>A potent inhibitor of hCEs</td>
</tr>
<tr>
<td>LPA</td>
<td>A selective inhibitor of hCE2</td>
</tr>
<tr>
<td>HA</td>
<td>A selective inhibitor of AChE</td>
</tr>
<tr>
<td>EDTA</td>
<td>A selective inhibitor of PON</td>
</tr>
</tbody>
</table>

Table S2 Kinetic parameters for DME hydrolysis in different enzyme sources

<table>
<thead>
<tr>
<th>Enzyme sources</th>
<th>Vmax (µmol·min⁻¹·mg⁻¹)</th>
<th>Km (µM)</th>
<th>CLint (L·min⁻¹·mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLM</td>
<td>1441±69</td>
<td>3.60±0.65</td>
<td>400.27</td>
</tr>
<tr>
<td>hCE1</td>
<td>1208±38</td>
<td>4.51±0.50</td>
<td>268.44</td>
</tr>
</tbody>
</table>
Scheme S1 The synthesis procedure of DME

Fig. S1 Luminescence responses of D-luciferin, DME with or without hCE1 pre-incubation.

Fig. S2 Effects of pH values on reaction system.

(a) DME was incubated in PBS with different pH values at 37 °C for 20 min, then luminescence were measured as described above. (b) D-luciferin was incubated in PBS with different pH values at 37 °C for 20 min, then luminescence were measured as described above. (c) hCE1 and DME in PBS with different pH values at 37 °C for 20 min then luminescence were measured as described above. The measurements were performed in
KH$_2$PO$_4$-K$_2$HPO$_4$ (PBS) buffer with different pH values.

Fig. S3 (a) Representative LC-UV chromatograms of DME (3 μM) incubation samples at 37 °C, (1) DME in PBS for 60 min; (2) DME in hCE1 (10 μg·mL$^{-1}$) for 20 min; (3) standard D-luciferin in PBS. Mass spectra of DME with the quasi-molecular ion peak $m/z = 295$ monitored under positive mode (b); and with the quasi-molecular ion peak $m/z = 293$ monitored under negative mode (c). Mass spectra of its hydrolyzed product D-luciferin with the quasi-molecular ion peak $m/z = 281$ monitored under positive mode (d); and with the
Fig. S4 The effects of different endogenous matrix on the enzymatic activities of hCE1.

hCE1 was pre-incubated with different matrix (common amino acids and metal ions) for 10 min, then DME (3 µM) was added to reaction mix and incubated for 10 min. hCE1 activity were determined by measurement the production of D-luciferin. 1, PBS; 2, Glu; 3, Lys; 4, Gln; 5, Ser; 6, Cys; 7, Vitamin c; 8, Gly; 9, Arg; 10, GSH; 11, Myristic acid; 12, Tyr; 13, Trp; 14, Glucose; 15, Bilirubin; 16, Cu²⁺; 17, Ca²⁺; 18, Fe³⁺; 19, Mn²⁺; 20, Co²⁺; 21, Mg²⁺; 22, Zn²⁺; 23, K⁺.
**Fig. S5** Dose-inhibition curves of BNPP (a) and bavachinin (b) on DME (3 μM) hydrolysis in hCE1 and HLM, respectively.
Fig. S6 Michaelis-Menten kinetic plots of DME hydrolysis in hCE1 (a) and HLM (b).
**Fig. S7** The effects of selective esterase inhibitors (100 µM) on DME hydrolysis in different tissue microsomes.

**Fig. S8** The effects of selective esterase inhibitors (100 µM) on DME hydrolysis in different tumor cells S9 fractions.
**Fig. S9** Cytotoxicity of DME in SKOV-3-Luc+ cells.

**Fig. S10** The effects of DMSO concentrations on the hydrolytic rates of DME (3 μM) upon addition of hCE1.
**Fig. S11** Bioluminescence intensity as a function of time after D-luciferin addition into culture medium of luciferase-transduced SKOV-3 cells.

**Fig. S12** (a) Bioluminescence intensity as a function of time upon DME addition in luciferase-transduced SKOV-3 cells. (b) Correlation between cell number per well and bioluminescence intensity for luciferase-transduced SKOV-3 cells. The measured intensity of bioluminescence was directly proportional to the number of cells.
Fig. S13 Inhibitory assays with BNPP on the live SKOV3-Luc+ cells. Bioluminescence imaging live SKOV3-Luc+ cells incubated with DMSO (1%, V/V) (a); and BNPP (100 µM) (b); (c) images of SKOV3-Luc+ cells pretreated with BNPP (100 µM) for 20 min followed by incubation with DME (50 µM); (d) images of SKOV3-Luc+ cells incubated with DME (50 µM).
**Fig. S14** $^1$H NMR (700 MHz, DMSO) spectrum of 2.

**Fig. S15** $^{13}$C NMR (700 MHz, DMSO) spectrum of 2.
Fig. S16 $^1$H NMR (700 MHz, DMSO) spectrum of DME.

Fig. S17 $^{13}$C NMR (700 MHz, DMSO) spectrum of DME.
References
