Supplementary Information

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

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Materials and methods

Materials

Human serum albumin (HSA), recombinant human acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) were obtained from Sigma-Aldrich (St. Louis, MO, USA). 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). cDNA-expressed recombinant human carboxylesterases including hCE1 and hCE2 were obtained from BD Biosciences (MA, USA). Human paraoxonases including PON1 (v13111203) and PON2 (v13111202) were obtained from bioworld (USA). Pooled human liver microsomes (HLMs) prepared from male Mongulia, and a panel of HLMs from twelve individuals (male Mongulia) were obtained from Research Institute for Liver Diseases (Shanghai, China). All microsomal samples and human enzymes were stored at -80 ºC until use. Millipore water (Millipore, USA) was used throughout. All other reagents, fine chemicals and LC solvents with the highest grade commercially available were obtained from J&K Chemical Ltd. (China) and Tedia (USA).

Synthesis and structural characterization of TCFB

To a solution of 0.5 mmol **TCF** and Et_3N (0.625 mmol) in 10 mL of DMF, benzoyl chloride (0.6 mmol, mixed with 5 mL of CH_2Cl_2) 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: 3, v/v) to afford 30.6 mg (15.1%) of **TCFB** as a red solid. The structure of **TCFB** was confirmed by ^{1}H-NMR, ^{13}C-NMR and HRMS spectroscopy (Fig. S1-S3, ESI†), and the data are as follows: ^{1}H NMR (400 MHz, DMSO) δ 8.16 (d, J = 7.9 Hz, 2H), 8.05 (d, J = 8.5 Hz, 2H), 7.97 (d, J = 16.5 Hz, 1H), 7.78 (t, J = 7.2 Hz, 1H), 7.63 (t, J = 7.7 Hz, 2H), 7.48 (d, J = 8.4 Hz, 2H), 7.26 (d, J = 16.5 Hz, 1H), 1.82 (s, 6H). ^{13}C NMR (100 MHz, DMSO) δ 177.54, 175.57, 164.74, 153.91, 146.65, 134.73, 132.73, 131.34, 130.36, 129.50, 129.10, 123.36, 116.06, 113.09, 112.26, 111.26, 100.14, 99.95, 55.08, 25.56. HRMS (ESI positive) calcd for $[M+Na]^+$ 430.1162, found 430.1147.

Instruments

^{1}H NMR and ^{13}C NMR spectra were recorded using Bruker Avance II (400 MHz) spectrometer with chemical shifts reported as ppm (in DMSO; TMS as internal standard). Accurate mass detection was measured on Hybrid Ion Trap-Orbitrap Mass Spectrometer (LTQ Orbitrap XL, Thermo). Absorption spectra and fluorescence emission/excitation spectra were measured on Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek). The hydrolysis supernatants were determined using a Shimadzu UFLC system coupled with a diode array detector (DAD) and a mass spectrometer (2010EV, Shimadzu, Japan). A stock solution of **TCFB** (1 mM) was prepared in DMSO and stored at -80 °C for future using. Stock solutions (5 mg/mL) of enzymes were prepared in phosphate buffer (pH=7.4) and stored at -80 °C.

Incubation conditions

The incubation mixture, with a total volume of 200 μL, consisted of 100 mM potassium phosphate buffer (pH=7.4), and human liver microsomes or other mentioned esterases. In all experiments, **TCFB** (1 mM dissolved in DMSO previously) was serially diluted to the required concentrations and the final concentration of DMSO did not exceed 2% (v/v) in the mixture. After pre-incubation at 37 ºC for 30 min, the reaction was initiated by adding **TCFB** and further incubated at 37 ºC in a shaking water bath. The reaction was terminated by the addition of ice-cold acetonitrile (equal volume of incubation mixture, 200 μL). Aliquots of supernatant were taken for further analysis. Control incubations without enzyme sources were carried out to ensure that metabolites formation was enzyme dependent. All incubations throughout the study were performed in duplicate with S.E.M. values generally below 10%.

LC-DAD-ESI-MS analysis

TCFB and its hydrolytic metabolite in hCE2 were identified by using LC-DAD-ESI-MS. The incubation conditions and procedures were used as mentioned above. The supernatants were analyzed using a Shimadzu UFLC system coupled with diode array detector and mass spectrometer. A Shim-Pack XR-ODS (7.5 ×2.1 mm, 3 μm) analytical column kept at a

temperature of 40 °C was used. The mobile phase consisted of CH_3CN (A) and water containing 0.2% formic acid (B) using a flow rate at 0.4 mL/min. **TCFB** and its hydrolytic metabolite can be quantified by using this LC-UV method monitored at 402 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 negative 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 and the drying gas (N_2) pressure was set at 0.06 MPa. Data processing was performed using the software LC-MS Solution version 3.41 (Shimadzu, Kyoto, Japan).

Determination of the quantum efficiency of fluorescence

For determination of the quantum efficiency of fluorescence (Φ_{fl}), rhodamine B in ethanol (Φ_{fl}=0.89) was used as a standard. Values were calculated according to the following equation. The quantum efficiencies of fluorescence were summarized in Table S1.

$\Phi_x/\Phi_s=[A_s/A_x][n_x^2/n_s^2][D_x/D_s]$

s: standard

x: sample

A: absorbance at the excitation wavelength

n: refractive index

D: area under the fluorescence spectra on an energy scale

Determination of the limit of detection

The detection limit was calculated based on the fluorescence titration. In the absence of hCE2, the fluorescence emission spectrum of **TCFB** was measured by five times and the standard deviation of blank measurement was achieved. To gain the slope, the fluorescence intensity at 612 nm was plotted as a concentration of hCE2. So the detection limit was calculated with the following equation:

Detection limit = $3\sigma/k$

Where σ is the standard deviation of blank measurement, k is the slope between the

fluorescence intensity at 612 nm versus hCE2 concentration.

Enzymatic reaction phenotyping assays

The specificity of **TCFB** (using a 20 μM solution in phosphate buffer, pH=7.4, 37 °C) toward various human enzymes with hydrolytic activity were investigated. Carboxylesterase (hCE1 and hCE2, 10 μg/mL), human serum albumin (HSA, 10 μg/mL), paraoxonase (PON1 and PON2, 10 μg/mL), bovine serum albumin (BSA, 10 μg/mL), acetylcholinesterase (AChE, 10 μg/mL), and butyrylcholinesterase (BChE, 20 U/L), were used to screen the enzyme(s) involved in **TCFB** hydrolysis. The incubations were carried out under the above mentioned incubation conditions. Fluorescence responses spectra were recorded after 30 min incubation at 37 °C. The excitation wavelength was 560 nm.

Chemical inhibition assays

TCFB hydrolysis in pooled HLM, HIM and hCE2 in the absence or presence of selective esterase inhibitors was measured to verify the involved enzyme(s) for this biotransformation. Briefly, **TCFB** (20 μM) was incubated in HLM (20 μg/mL), HIM (20 μg/mL) or hCE2 (10 μg/mL) in the absence (control) or presence of known selective esterase inhibitors, including BNPP (a potent inhibitor of hCEs, 50 μM), LPA (a selective inhibitor of hCE2, 50 μM), EDTA (a selective inhibitor of PON, 100 μM) and HA (a selective inhibitor of AChE, 100 μM). Each inhibitor was pre-incubated within the reaction mixtures (200 μL total volume) containing HLM at 37 °C for 10 min, then the reactions were initiated by adding **TCFB** into the enzyme mixtures. IC_{50} values of LPA for HLM (20 μg/mL), HIM (20 μg/mL) and hCE2 (10 μg/mL) were determined by incubating **TCFB** (20 μM) with varying concentrations of LPA (0.001-100 μM). Reaction inhibition was expressed as percent decrease in fluorescent intensity at 612 nm. Data were fit to log (inhibitor) vs. normalized response - Variable slope equation using GraphPad Prism 6.0 (San Diego, CA).

Enzyme kinetics analysis

Briefly, HLM (20 μg/mL), HIM (20 μg/mL) or hCE2 (10 μg/mL) was incubated with **TCFB** (2-60 μM) in 200 μL of 100 mM phosphate buffer solution (pH 7.4), respectively. Incubation

time and protein concentration were optimized within a linear range response. The reactions were initiated by adding serial concentrations of **TCFB** to pre-incubated enzyme mixture. After 30 min incubation, reaction was terminated by adding equal volume of icy acetonitrile. The formation of metabolite was determined bymeasuring the fluorescence intensity of **TCF** at 612 nm. Kinetic parameters (K_m and V_{max}) were determined by nonlinear regression analysis using the Michaelis-Menten equation (GraphPad Prism).

Correlation studies

In order to evaluate the selectivity of **TCFB** in human biological samples, the formation rates of **TCF** in HLMs were compared with the catalytic activities of hCE2, respectively. Fluorescein diacetate (FD) was used as a probe substrate for hCE2. Firstly, the reaction rates of **TCFB** hydrolysis, FD hydrolysis were determined in a panel of HLMs prepared from 12 individual donors, respectively. All hydrolysis reactions were carried out in PBS buffer (0.1 M, pH 7.4) at 37 °C in a total volume of 200 μL. The hydrolysis of **TCFB** (final concentration, 20 μM) was performed in HLM (final protein concentration, 10 μg/mL) for 30 min. FD (final concentration, 10 μM) was incubated with HLM (final protein concentration, 5 μg/mL) for 30 min. All the incubations were terminated with equal volume (200 μL) of icy acetonitrile. The hydrolytic product of FD (fluorescein) was measured by Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek). ($\lambda_{em/ex}$=480 /520 nm) Then the rates of **TCFB** hydrolysis in 12 individual HLMs were compared with the rates of FD hydrolysis. The correlation parameter was expressed by the linear regression coefficient (R^2). $P<0.005$ was considered statistically significant.

Molecular Dynamics and molecular docking method

The initial structure of hCE2 was modeled in terms of the protein structure of human liver carboxylesterase 1 (hCE1, PDB ID: 1YA4) using the modeller (version 9) program.[1] The protein structure of hCE2 was further refined by performing molecular dynamics (MD) simulations using NAMD 2.9 software.[2] The CHARMM 27 force field was used to represent the protein structure. The structure was solvated in an 86×92×94 $Å^3$ box with TIP3P water model. The distance between any atom of protein and the border of water box was at least 12

Å. Thirteen Na^+ ions were added to neutralize the system. Periodic boundary conditions were applied in three dimensions. The long-range electrostatic interactions were treated using the PME method. The integration time step is 2 fs. The system was first energy minimized for 10 000 steps with the protein structure fixed. Subsequently, the system was heated from 0 to 310 K in 100 ps, followed by an equilibration at 310 K for 100 ps. Langevin dynamics and Langevin piston methods were used to maintain the temperature at 310 K and pressure at 1 bar. The production MD simulations were run for 10 ns. The final 10-ns protein structure was used for docking with AutoDock software.[3] The ligand (please specified which ligand) parameters were obtained using the PRODRG Server (http://davapc1.bioch.dundee.ac.uk/cgi-bin/prodrg).[4] The Gasteiger charge was calculated for the hCE2 protein structure. The grid box was sufficiently large to cover the whole protein, and the ligand (**TCFB**) was blindly docked into the protein using the Lamarckian generic algorithm. The other default parameters in AutoDock were applied for docking. The protein structure of hCE2 is available upon request from the corresponding author.

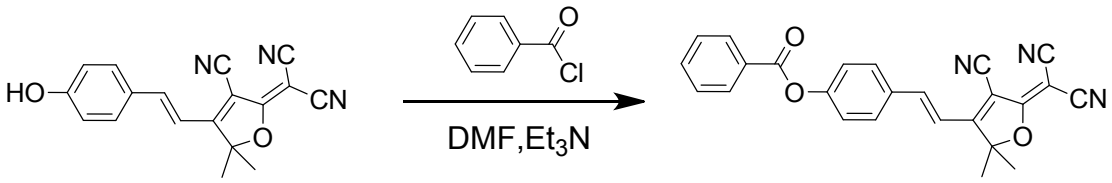
Cytotoxicity assays

Cell viability was investigated by Sulforhodamine B (SRB) method. A549 cells (5×10^4/mL, 200 μL) were seeded in 96-well plate and maintained at 37 °C in a 5% CO_2 incubator for 24 h. Then the cells were incubated with different concentrations of **TCFB** (0.001, 0.01, 0.1, 1, 10, 50, 100 μM) for another 4 h. Icy trichloroacetic acid (10%, w/v, 100 μL) were added into the adherent cells and fixed in 4 °C for 1h. SRB solution (4%, 40 μL) was added into the overnight dried cell plate, and staining at 37 °C for 30 min. After washed by 1% (v/v) acetic acid solution, tris buffer (100 μL) was added and vortex mixed. The absorbance was measured at 570 nm.

Confocal fluorescence imaging in living A549 cells

Supplemented with fetal bovine serum of 10%, cells were grown in Dulbecco's modification of Eagle's medium Dulbecco (DMEM/high: with 4500 mg/L Glucose, 4.0 mM L-Glutamine, and 110 mg/L Sodium Pyruvate). In 12-well culture plate, celled were seeded, incubated in a CO_2 humidified incubator of 5% at 37 °C overnight. Washed twice with FBS-free DMEM,

the adherent cells were incubated with/without 100 μM LPA (preparing in FBS-free DMEM) for 30 min at 37 °C in CO_2 incubator of 5%. At a 50 μM final concentration, stock solution of probe **TCFB** (5 mM) in DMSO was diluted into the cell culture media (FBS free). The cells were then incubated at 37 °C for another 30 min, and then washed with PBS (pH 7.4) for three times, and then imaged on confocal microscope (Olympus, FV1000).



Scheme S1 The synthesis procedure of **TCFB**.

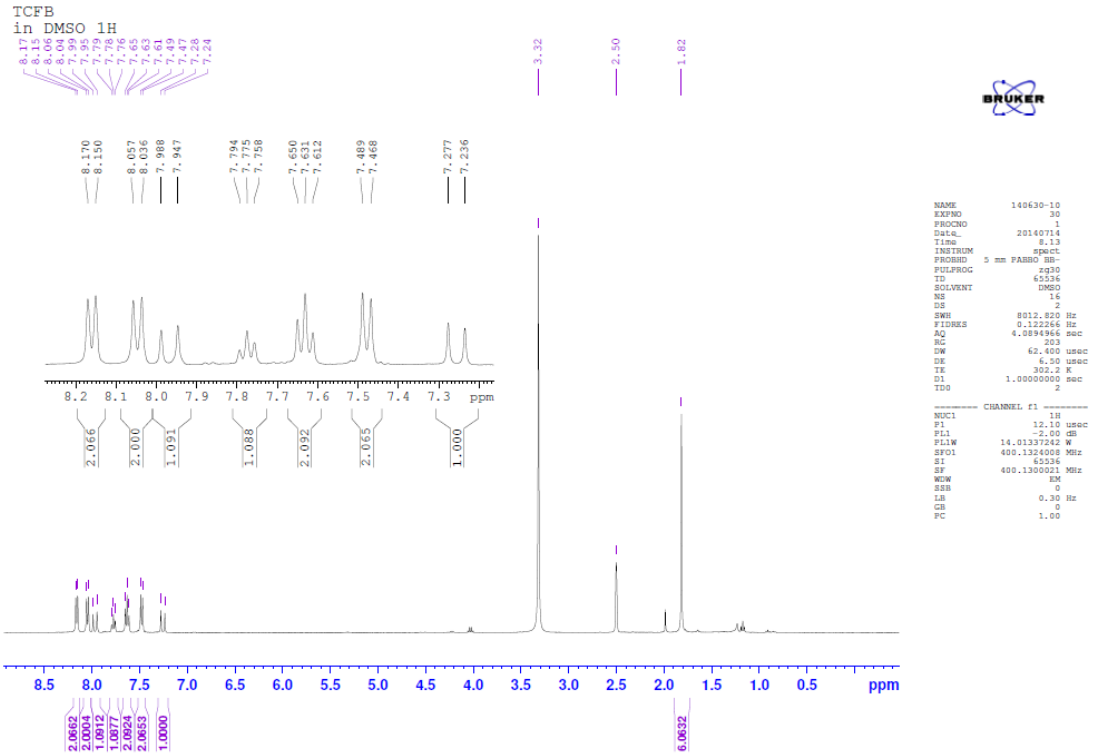


Fig. S1 ^{1}H NMR (400 MHz, DMSO) spectrum of **TCFB**.

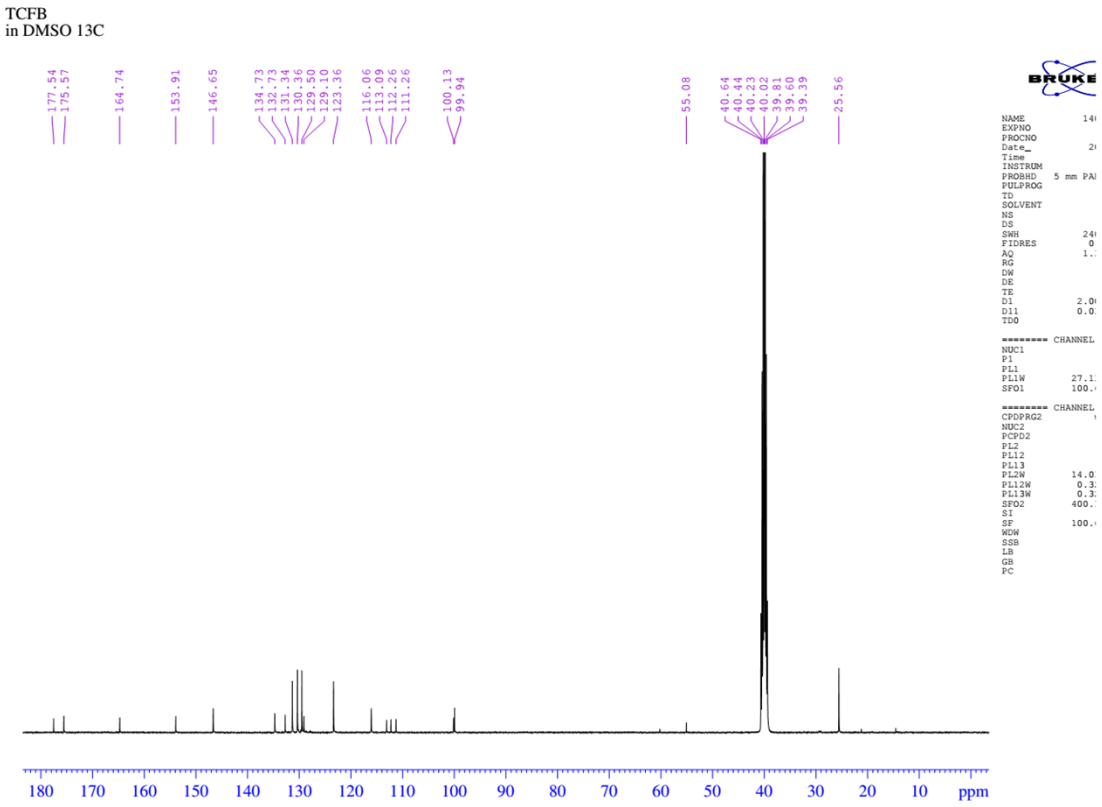


Fig.S2 ^{13}C NMR (100 MHz, DMSO) spectrum of **TCFB**.

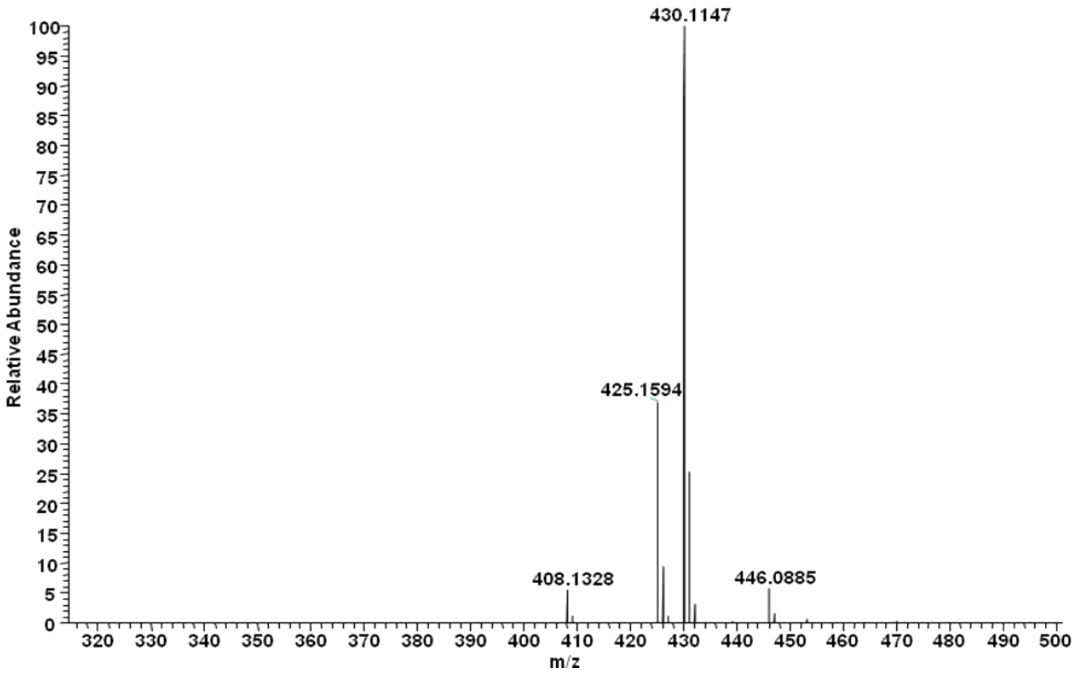


Fig. S3 HRMS spectrum of **TCFB**.

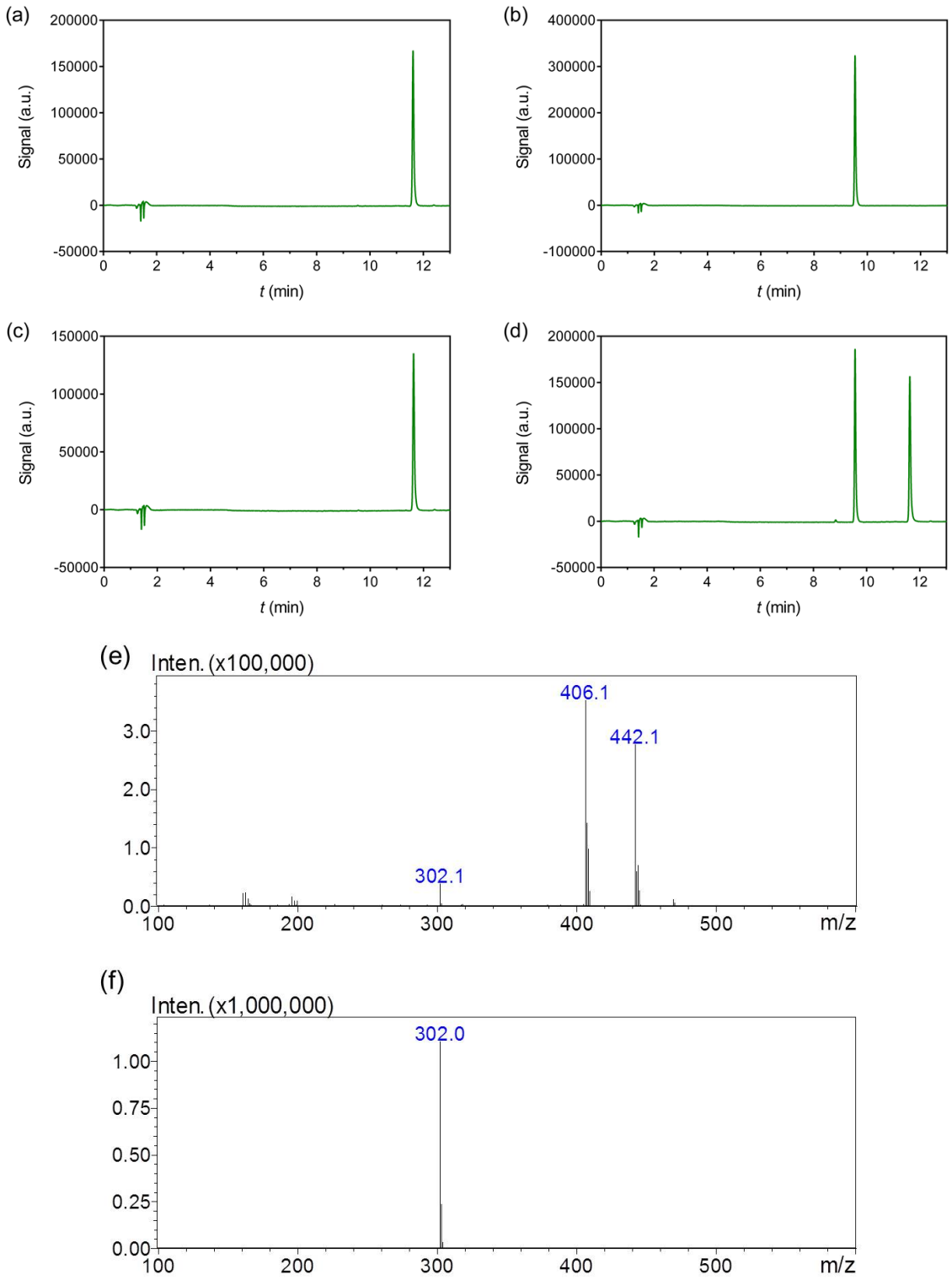


Fig. S4 LC-ESI-MS analysis of the reaction mixtures of **TCFB**. LC chromatograms of the incubation samples (a. **TCFB** standard, b. **TCF** standard, c. **TCFB** (20 μM) incubation in phosphate buffer for 30 min, d. **TCFB** (20 μM) incubation with hCE2 (10 μg/mL) for 30 min. The detection wavelength was set at 402 nm. The mass spectra of **TCFB** (e) and its metabolite (f) were recorded under negative ion mode.

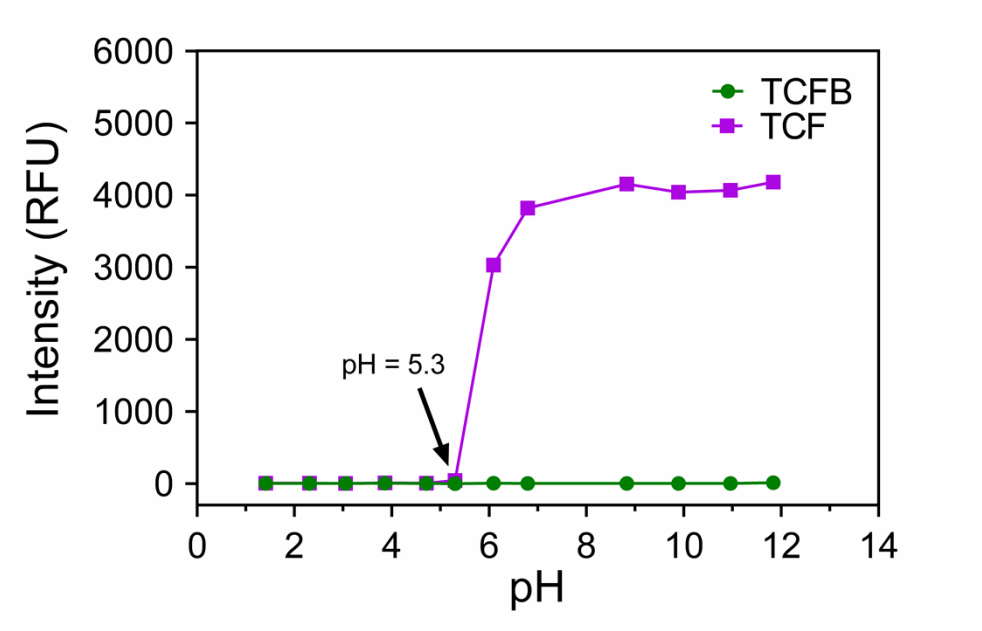


Fig. S5. Effects of pH values on the fluorescence intensity at 612 nm of **TCFB** (20 μM) and its metabolite **TCF** (20 μM). The measurements were performed in KCl-HCl buffer-acetonitrile (1: 1, v/v) with different pH values adjusted by KOH. Excitation wavelength was 560 nm.

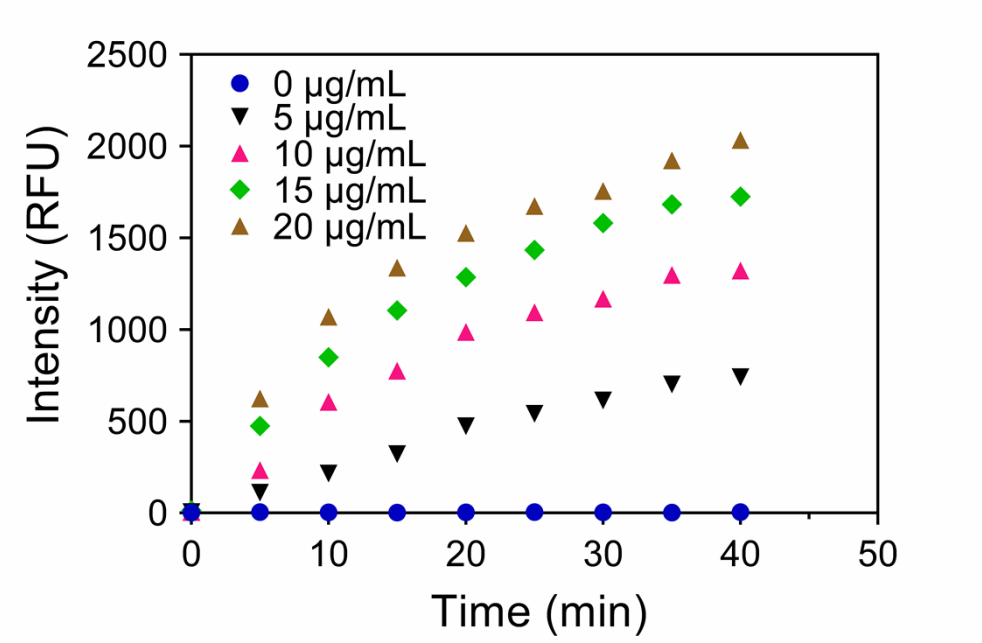


Fig. S6 Time-dependent fluorescence intensity at 612 nm changes of **TCFB** (20 μM) with different concentrations (0, 5, 10, 15, 20 μg/mL) of hCE2 at 37 °C. Excitation wavelength was 560 nm.

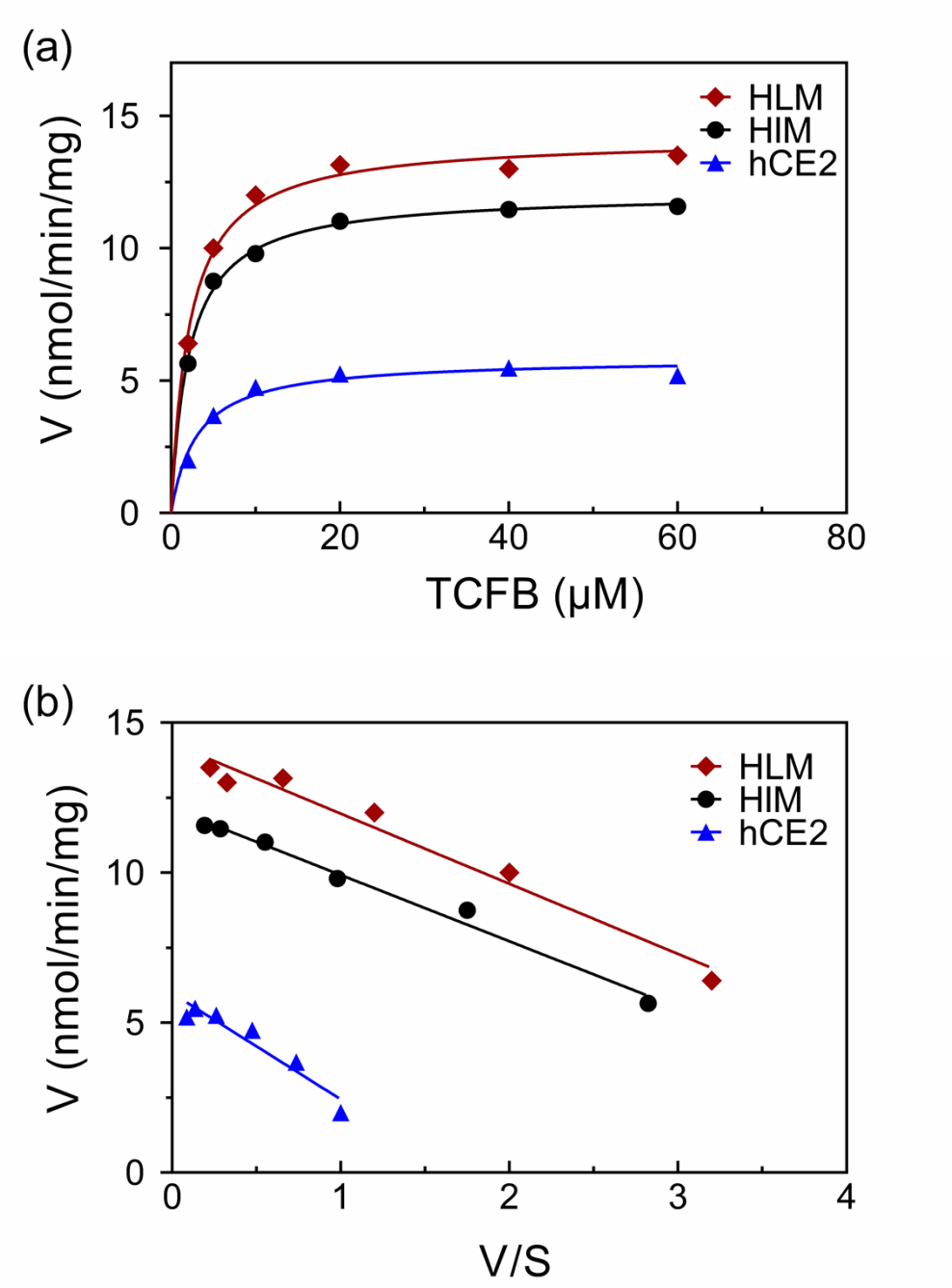


Fig. S7 Kinetic plot (a) and Eadie-Hofstee plot (b) of **TCFB** hydrolysis in HIM, HLM and hCE2. Catalytic activity of hCE2 was determined by use of the fluorescence intensity at 612 nm. The excitation wavelength was 560 nm. The Michaelis-Menten kinetic parameters were summarized in Table S2.

Table S1 The quantum efficiencies of fluorescence of **TCFB** and **TCF** in PBS-acetonitrile (V: V=1: 1, pH 7.4).

Compound	λ_{ex} (nm)	λ_{em} (nm)	Φ_{fl}[a]
TCFB	460	/	＜0.00001
TCF	530	612	0.0022

[a] Quantum efficiencies were determined by using rhodamine B in ethanol (Φ_{fl} = 0.89) as a standard.

Table S2 Kinetic parameters of **TCFB** hydrolysis determined in different enzyme resources. Each value was the mean ± SEM of determinations performed in duplicate.

Enzyme resources	k_{cat} nmol/min/mg protein	K_m μM	k_{cat}/K_m mL/min/mg protein
HLM (Pooled)	14.17 ± 0.29	2.19 ± 0.24	6.47
HIM (Pooled)	12.08 ±0.15	2.14 ±0.14	5.64
hCE2	5.84 ± 0.23	3.06 ± 0.57	1.91

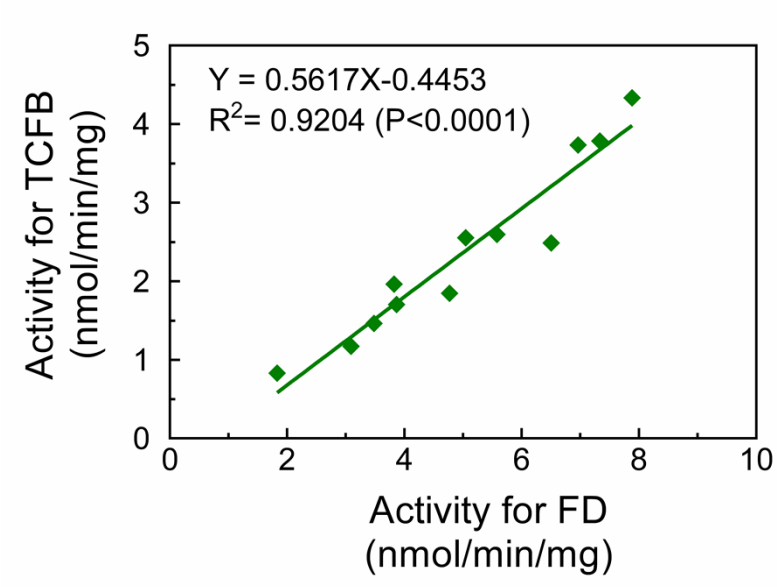


Fig. S8 Correlation analysis between FD (10 μM) and **TCFB** (20 μM) hydrolysis by HLMs. Experiments were performed in twelve individual HLMs (final protein concentration, 10 μg/mL) at 37 °C for 30 min. The activities for FD and **TCFB** were expressed by the formation rate of F ($\lambda_{ex/em}$=480/520 nm) and **TCF** ($\lambda_{ex/em}$=560/612 nm).

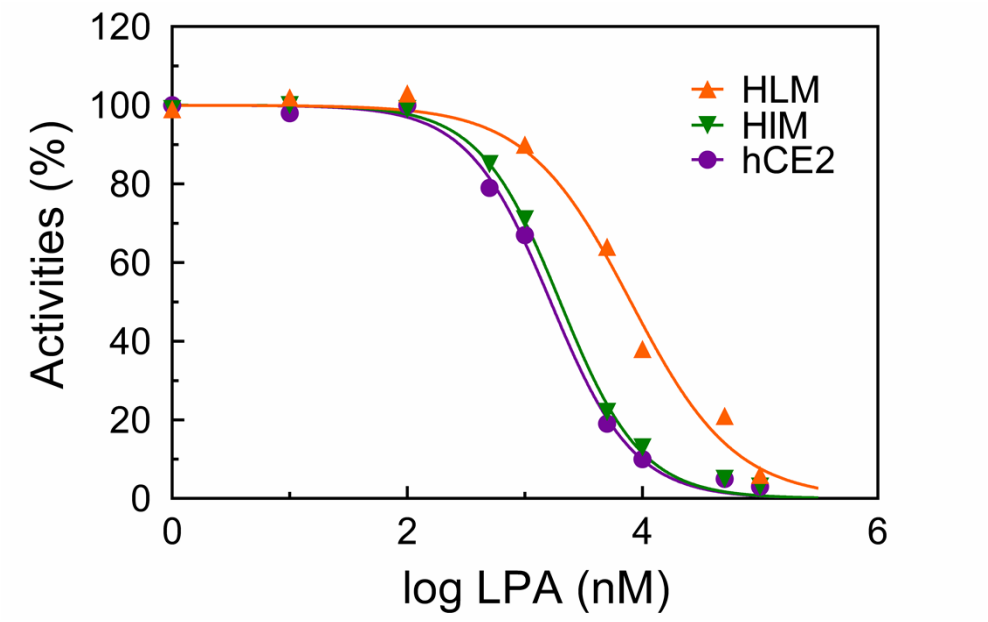


Fig. S9 Dose-inhibition curves of LPA (0.001-100 μM) on **TCFB** (20 μM) hydrolysis in HLM (20 μg/mL), HIM (20 μg/mL) and hCE2 (10 μg/mL). Reaction inhibition was expressed as percent decrease in fluorescent intensity at 612 nm. Data were fitting to log (I) vs. normalized response equation by using GraphPad Prism 6 to calculate the IC_{50} values. Excitation wavelength was 560 nm. The inhibition profiles and the inhibition capability of LPA in the mixed enzyme system (HLM and HIM) are very similar to those in hCE2, with the closed IC_{50} values of 7.96 μM, 1.99 μM, and 1.66 μM, respectively.

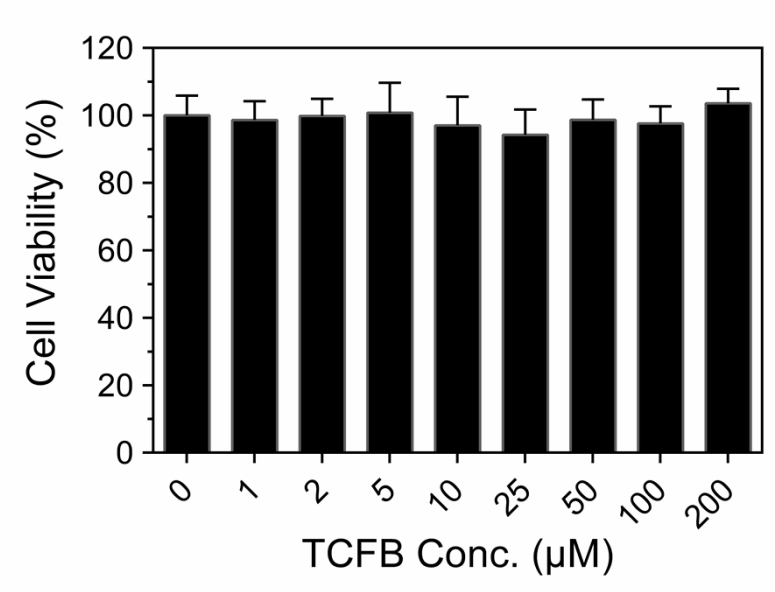


Fig. S10 Cell viability of A549 cells in the presence of different concentrations of **TCFB** was determined by using a standard SRB assay.

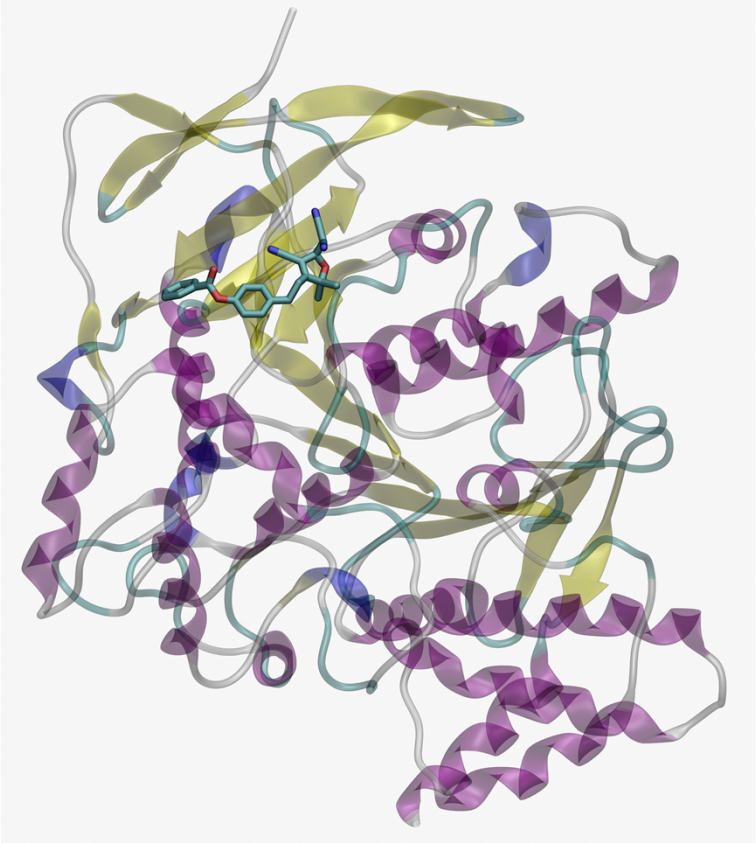


Fig. S11 The diagram of human liver carboxylesterase 1 (hCE1, PDB ID: 1YA4) docking with **TCFB**. The AutoDock software was used to dock the compound into the active site of hCE1.

References

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