Supporting Information

Drug-Target Residence Time Affects *in vivo* Target Occupancy through Multiple Pathways

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1. Supporting Figure

Figure S1 Pharmacokinetic profile of TPPU with blood concentration, which expressed as log scale, showed that there are three elimination phases and there is a very long gamma phase. In this study, our data suggest that the long gamma phase is due to TPPU binding selectively to sEH. Although it is possible that the long gamma phase might be an experimental artifact, our identical experiment with sEH KO mice that did not show the same long gamma phase, confirmed that the obtained result is real. Male mice (n=6) were treated by subcutaneous injection (0.3 mg/kg dissolved in PEG400, 100 to 110 μL of total injection volume). The data are mean ± standard error of the mean (SEM). The graphics and statistics were prepared by SigmaPlot (SysTat Software, San Jose, CA).
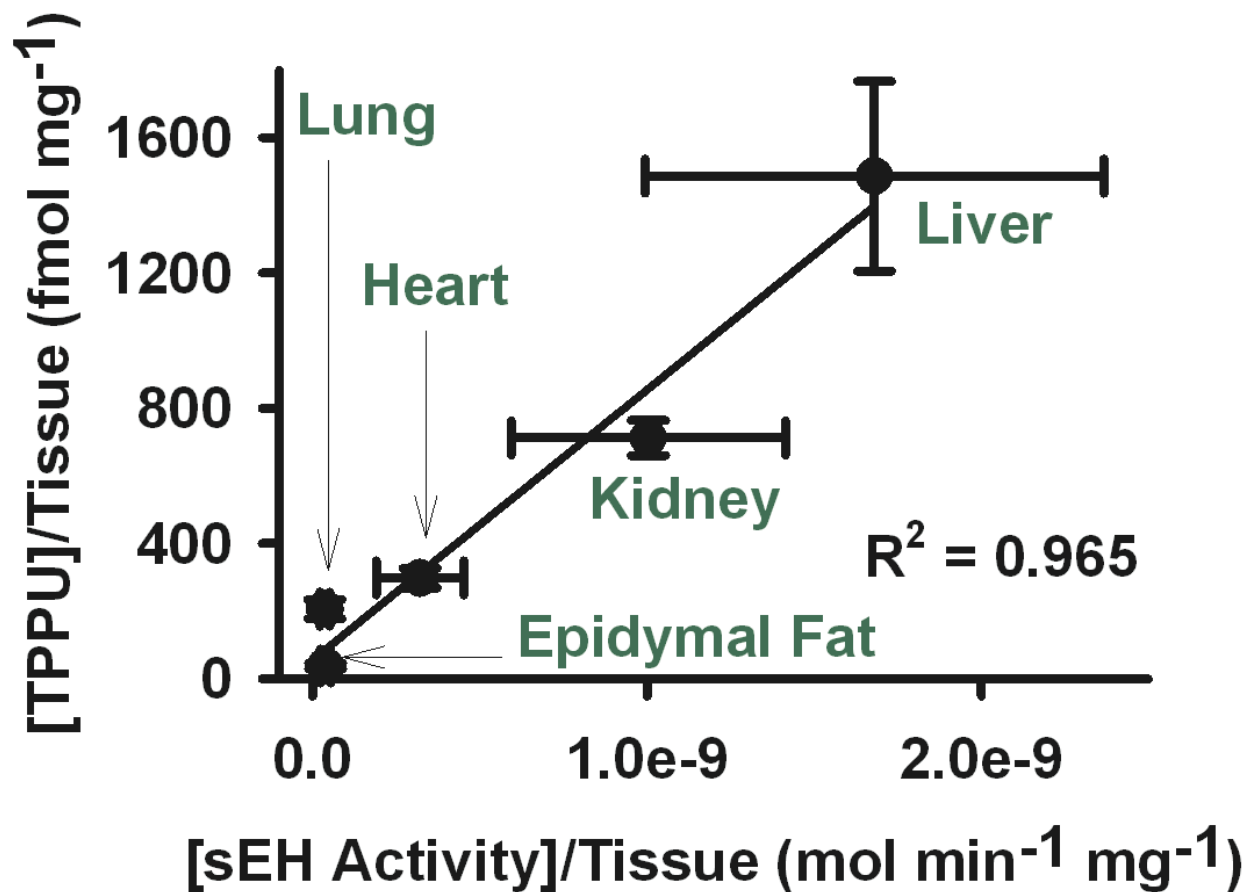
Figure S2 TPPU level at post-dosing day 7 correlated well with sEH activity in different tissues (Specific Tissues are indicated). R^2 was calculated based on the datum point close to the fitted line (black). The data are mean ± SEM.
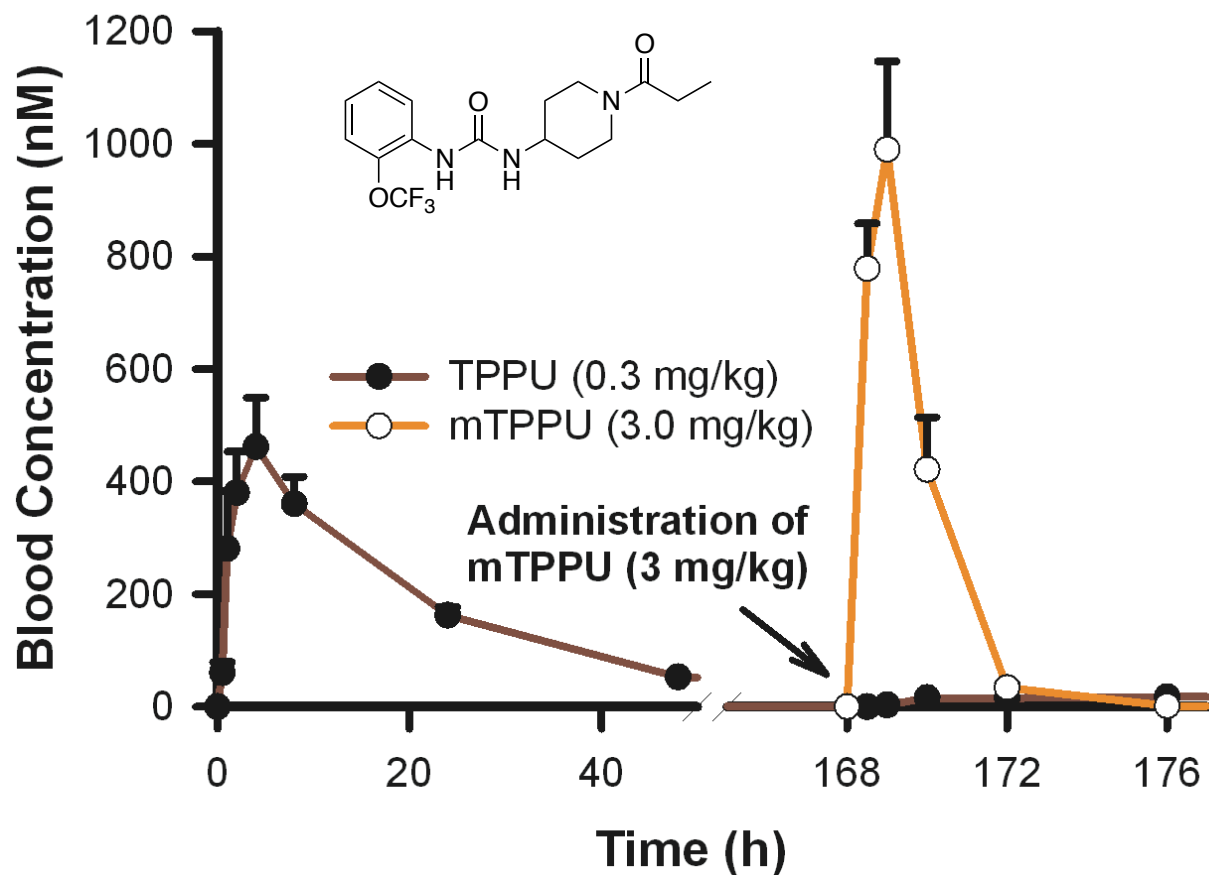
Figure S3 The pharmacokinetic profiles of *in vivo* displacement assay of TPPU (0.3 mg/kg) in WT mice with mTPPU (3 mg/kg) shows that the much less-potent mTPPU (IC$_{50}$ ≥ 10 μM) cannot displace the sEH bounded TPPU *in vivo*. The result suggests that the displacement peak reflects the inhibitor specifically bound to sEH. Male mice (n=6) were treated by subcutaneous injection (0.3 mg/kg TPPU or 3.0 mg/kg mTPPU dissolved in PEG400, 100 to 110 μL of total injection volume). The data are mean ± SEM. The graphics and statistics were prepared by SigmaPlot (SysTat Software, San Jose, CA).
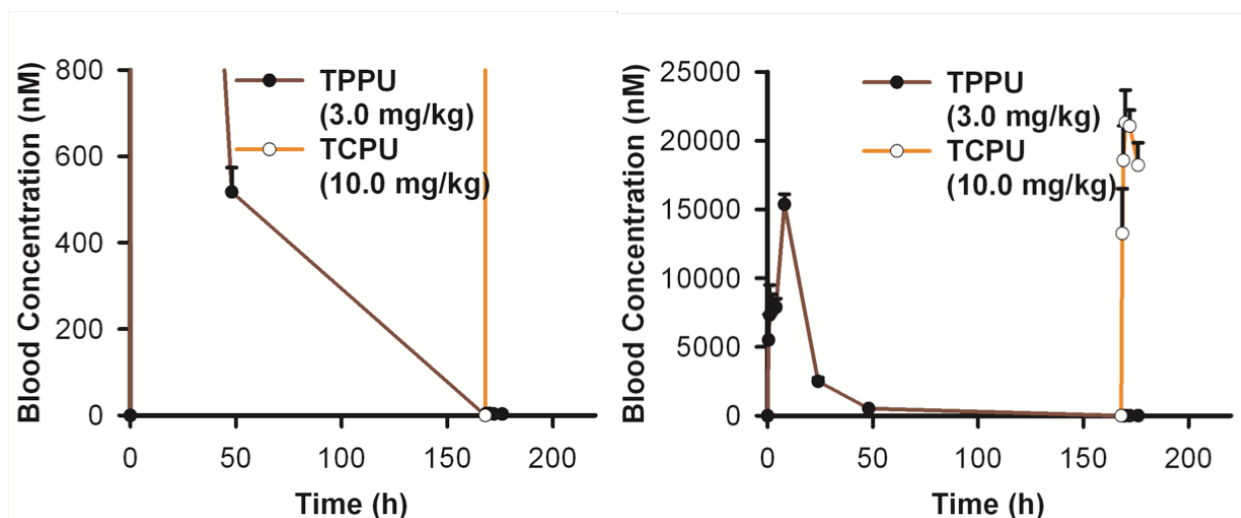
***Figure S4*** In sEH null mice, a dose of TCPU (10 mg/kg, orange line, open circle) displaces very minimum amounts of TPPU (3 mg/kg, red line, black circle) as compared to a similar experiment where a 3 mg/kg dose of TCPU was used (**Figure 2A**). **Left:** A zoom in figure of the PK profile of the *in vivo* displacement assay with a tested inhibitor, TPPU (3 mg/kg, red line, black circle), and the displacement inhibitor, TCPU (10 mg/kg, orange line, open circle), in sEH null mice. **Right:** A full scale figure of the PK profile of the *in vivo* displacement assay with a tested inhibitor, TPPU (3 mg/kg, red line, black circle), and a displacement inhibitor, TCPU (10 mg/kg, orange line, open circle), in sEH null mice. Male sEH null mice (n=6) were treated by subcutaneous injection (3 mg/kg TPPU or 10 mg/kg TCPU dissolved in PEG400, 100 to 110 µL of total injection volume). The data are mean ± SEM. The graphics and statistics were prepared by SigmaPlot (SysTat Software, San Jose, CA).
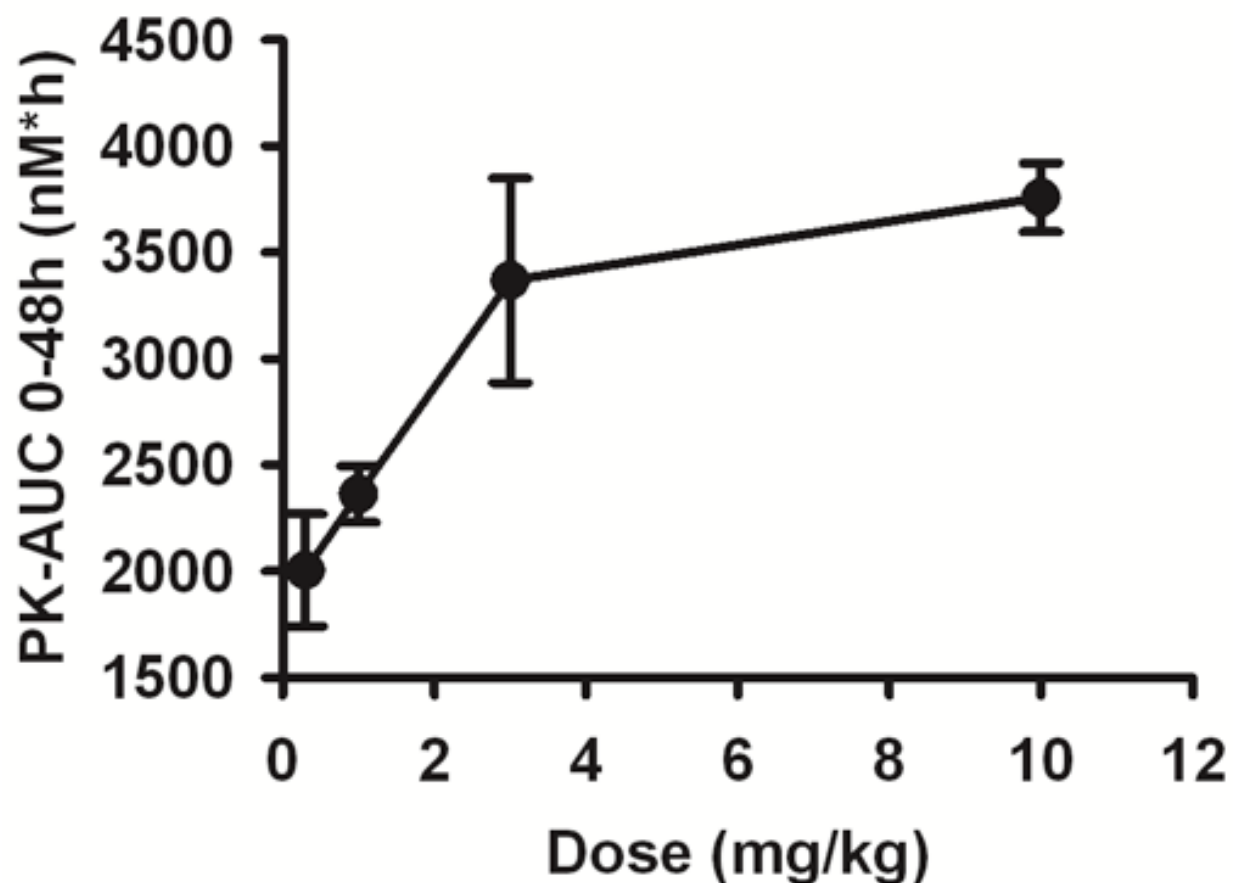
Figure S5 Determination of the optimum dose of TCPU, a displacement inhibitor B, to be used for the displacement of TPPU. The tested inhibitor for the *in vivo* displacement assay is 3 mg/kg based on our escalating dose study of displacement inhibitor B. Our data show that at 3 mg/kg of TCPU, the AUC of the 2nd displacement peak of TPPU reaches the maximum and further increasing the dose of TCPU to 10 mg/kg does does not increase the AUC of the 2nd displacement peak of TPPU. Male mice (n=3 to 6) were treated by subcutaneous injection (0.3 mg/kg of TPPU or 0.3 mg/kg to 10 mg/kg of TCPU dissolved in PEG400, 100 to 110 µL of total injection volume depends on the weight of the mouse). The data are mean ± SEM. The graphics and statistics were prepared by SigmaPlot (SysTat Software, San Jose, CA).
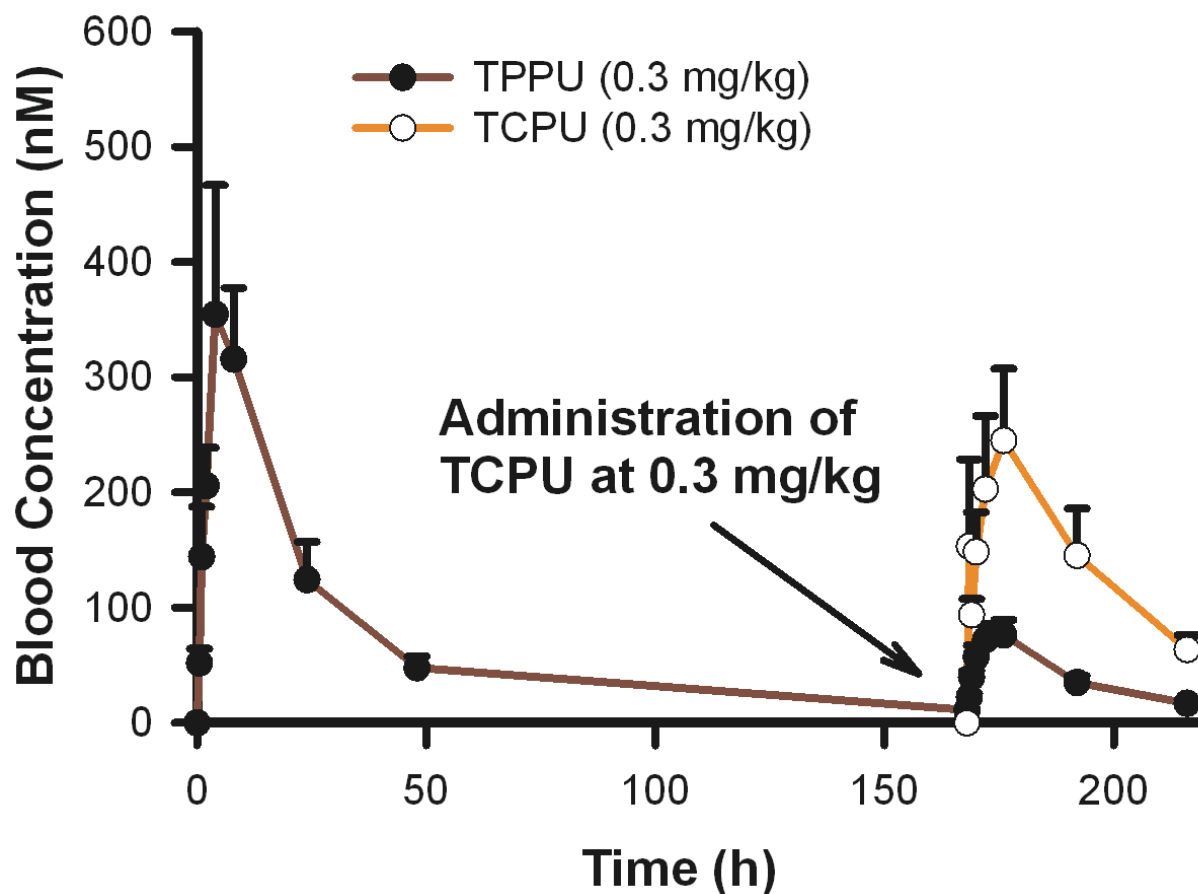
Figure S6 A lower dose of TCPU (0.3 mg/kg, orange line, open circle) displaces less TPPU (0.3 mg/kg, brown line, black circle) as compared to a similar experiment with high dose of TCPU (3 mg/kg) was used (**Figure 2A**). Male mice (n=6) were treated by subcutaneous injection (0.3 mg/kg TPPU or 0.3 mg/kg TCPU dissolved in PEG400, 100 to 110 µL of total injection volume). The data are mean ± SEM. The graphics and statistics were prepared by SigmaPlot (SysTat Software, San Jose, CA).
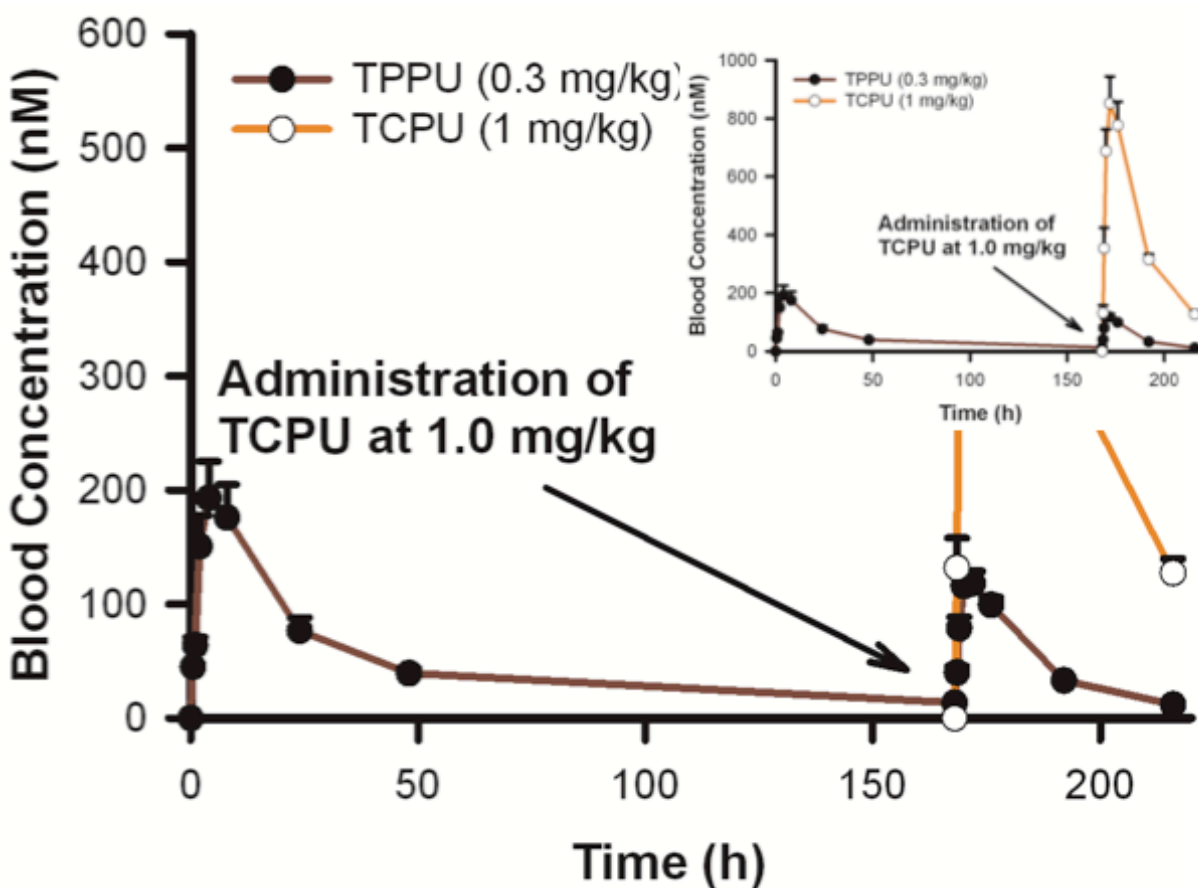
Figure S7 A lower dose of TCPU (1 mg/kg, orange line, open circle) displaces less TPPU (0.3 mg/kg, red line, black circle) as compared to a similar experiment where a higher dose of TCPU (3 mg/kg) was used (**Figure 2A**). Male mice (n= 6) were treated by subcutaneous injection (0.3 mg/kg or 1.0 mg/kg TPPU dissolved in PEG400, 100 to 110 µL of total injection volume). The pharmacokinetic profile of both TPPU (0.3 mg/kg) and TCPU (1.0 mg/kg) were included in the small figures. The data are mean ± SEM. The graphics and statistics were prepared by SigmaPlot (SysTat Software, San Jose, CA).
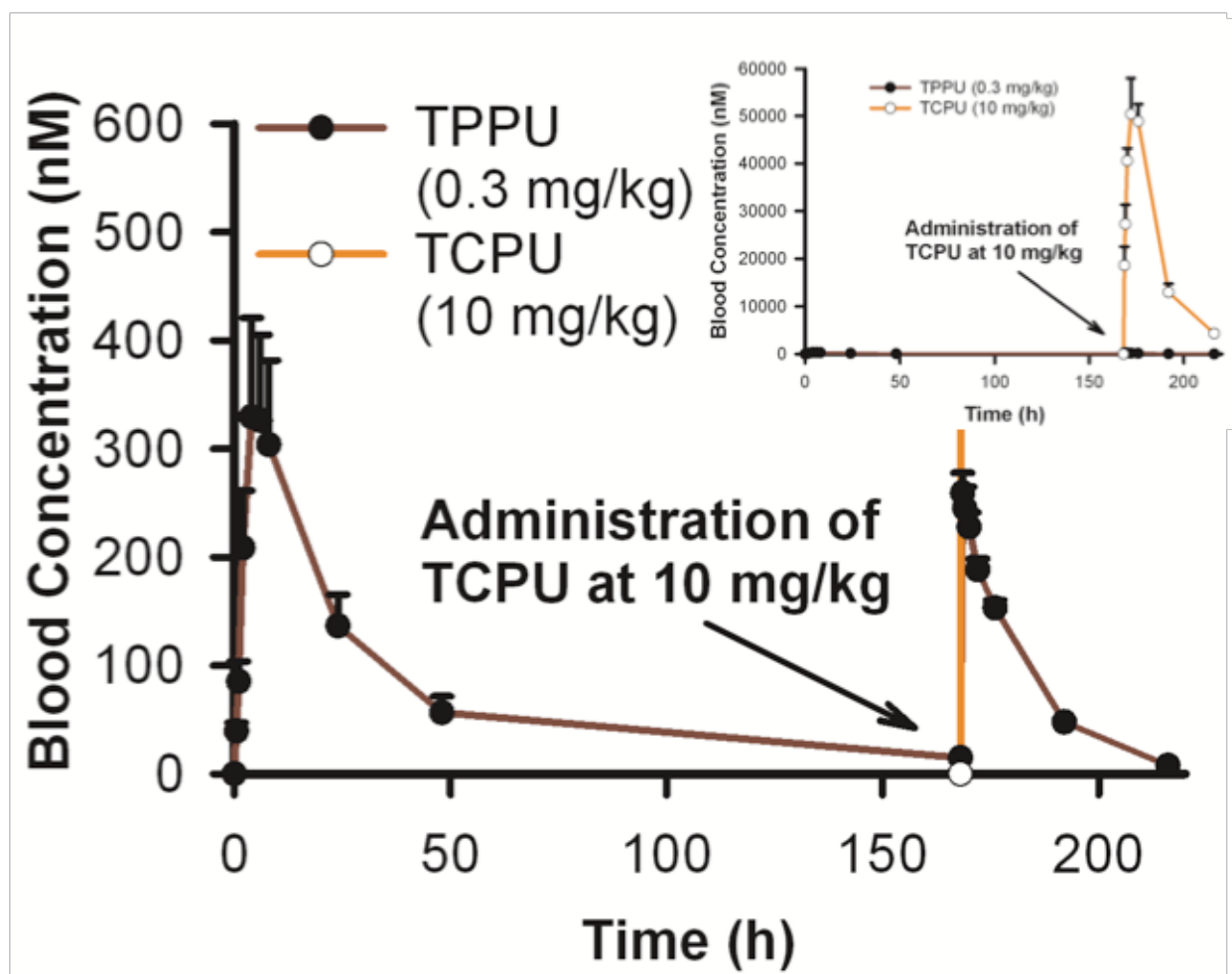
**Figure S8** *In vivo* displacement assay of TPPU (0.3 mg/kg) in WT mouse with TCPU (10 mg/kg). A higher dose of TCPU (10 mg/kg, orange line, open circle) displaces similar amount of TPPU (0.3 mg/kg, red line, black circle) as compared to a similar experiment with a lower dose of TCPU (3 mg/kg) was used (**Figure 2A**). Male mice (n= 6) were treated by subcutaneous injection (0.3 mg/kg TPPU or 10 mg/kg TCPU dissolved in PEG400, 100 to 110 μL of total injection volume). The pharmacokinetic profile of both TPPU (0.3 mg/kg) and TCPU (10 mg/kg) were included in the small figures. The data are mean ± SEM. The graphics and statistics were prepared by SigmaPlot (SysTat Software, San Jose, CA).
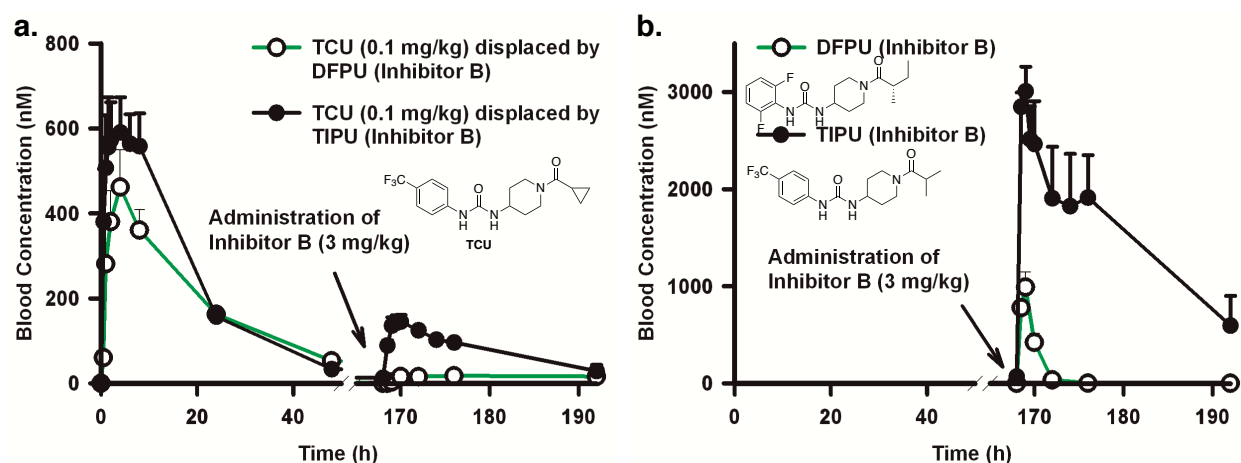
**Figure S9 a)** Our data from the *in vivo* displacement assay in rodents suggests that the displacement peak of TCU (black line, solid circle) showed at post-dosing time 168h after administration of a high dose of TIPU as an displacement inhibitor B is from the sEH-bound TCU. It is because the much less potent sEH inhibitors (≤10,000-fold less potent than TIPU) are unable to displace the sEH-bound TCU and the displacement peak was not observed (green line, open circle). Male rat (n=4) were administrated with TCU (0.3 mg/kg, PEG400:olelate rich safflower oil/ 1:4, 1mL, oral gavage) at time 0h. At post-dosing time 168h, DFPU (green line with open circle, n=4) and TIPU (black line with black circle, n=4) were administration (3 mg/kg, PEG400:olelate rich safflower oil/ 1:4, 1mL, oral gavage) **b)** A substantial amount of DFPU (green line with open circle, n=4) and TIPU (black line with black circle, n=4) were observed in blood after administration of a either high dose of DFPU (3 mg/kg, PEG400:olelate rich safflower oil/ 1:4, 1mL, oral gavage, green line, open circle) or TIPU (3 mg/kg, PEG400:olelate rich safflower oil/ 1:4, 1mL, oral gavage, black line, solid circle) at 168h from the same *in vivo* displacement assay in **Figure S9a**. The data are mean ± SEM. All the graphics and statistics were prepared by SigmaPlot (SysTat Software, San Jose, CA).
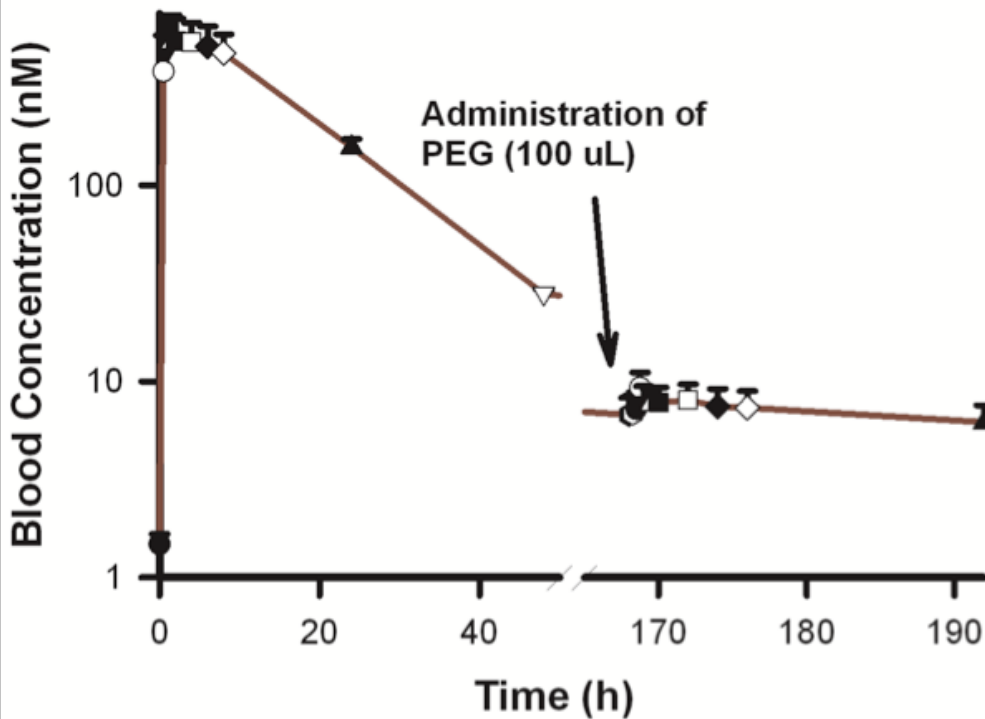
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in vivo Displacement Assay of
TCU (0.1 mg/kg) in Rat with PEG (100 uL)

Administration of
PEG (100 uL)

in vivo Displacement Assay of
TCU (0.1 mg/kg) in Rat with PEG (100 uL)

Administration of
PEG (100 uL)
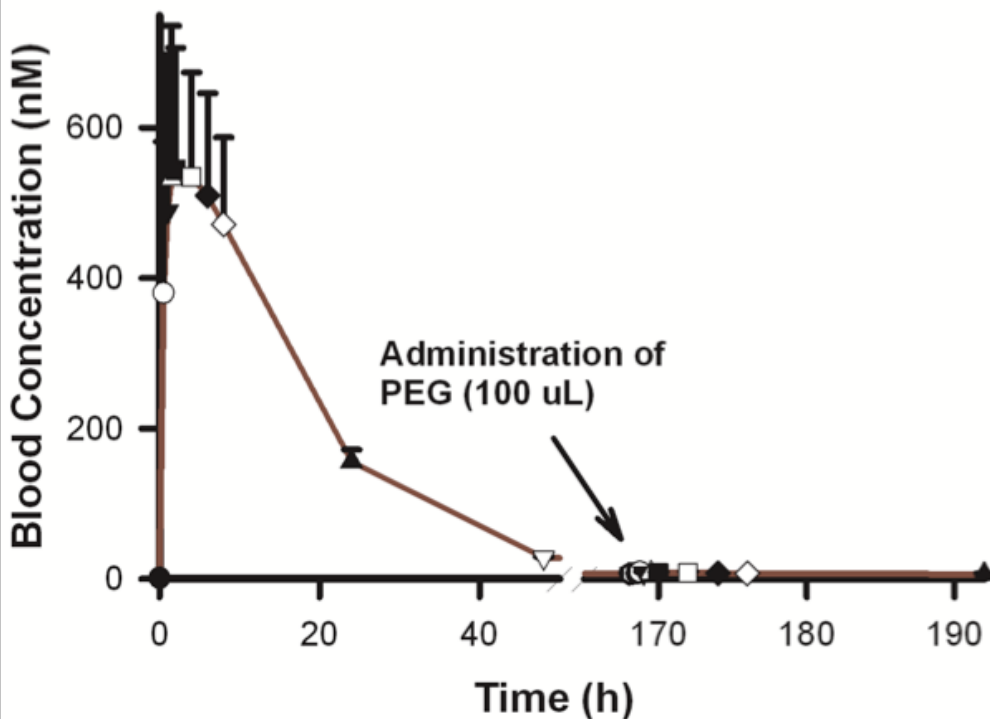
Figure S10 The *in vivo* displacement assay of TCU (0.1 mg/kg) with vehicle (PEG400) showed no 2nd displacement peak which indicating that the 2nd displacement peak is not due to the vehicle effect. The administration of PEG400 (PEG, vehicle) at 168h post-dosing of TPCU did not knock off any bound TPCU in Sprague Dawley Rat. Male rat (n=3) were treated by oral gavage (0.1 mg/kg of TPCU dissolved in PEG400 or pure PEG400, 100 ul of total injection volume). **Top:** The data in log scale. **Bottom:** the data in linear scale. The data are mean ± SEM. The graphics and statistics were prepared by SigmaPlot (SysTat Software, San Jose, CA).
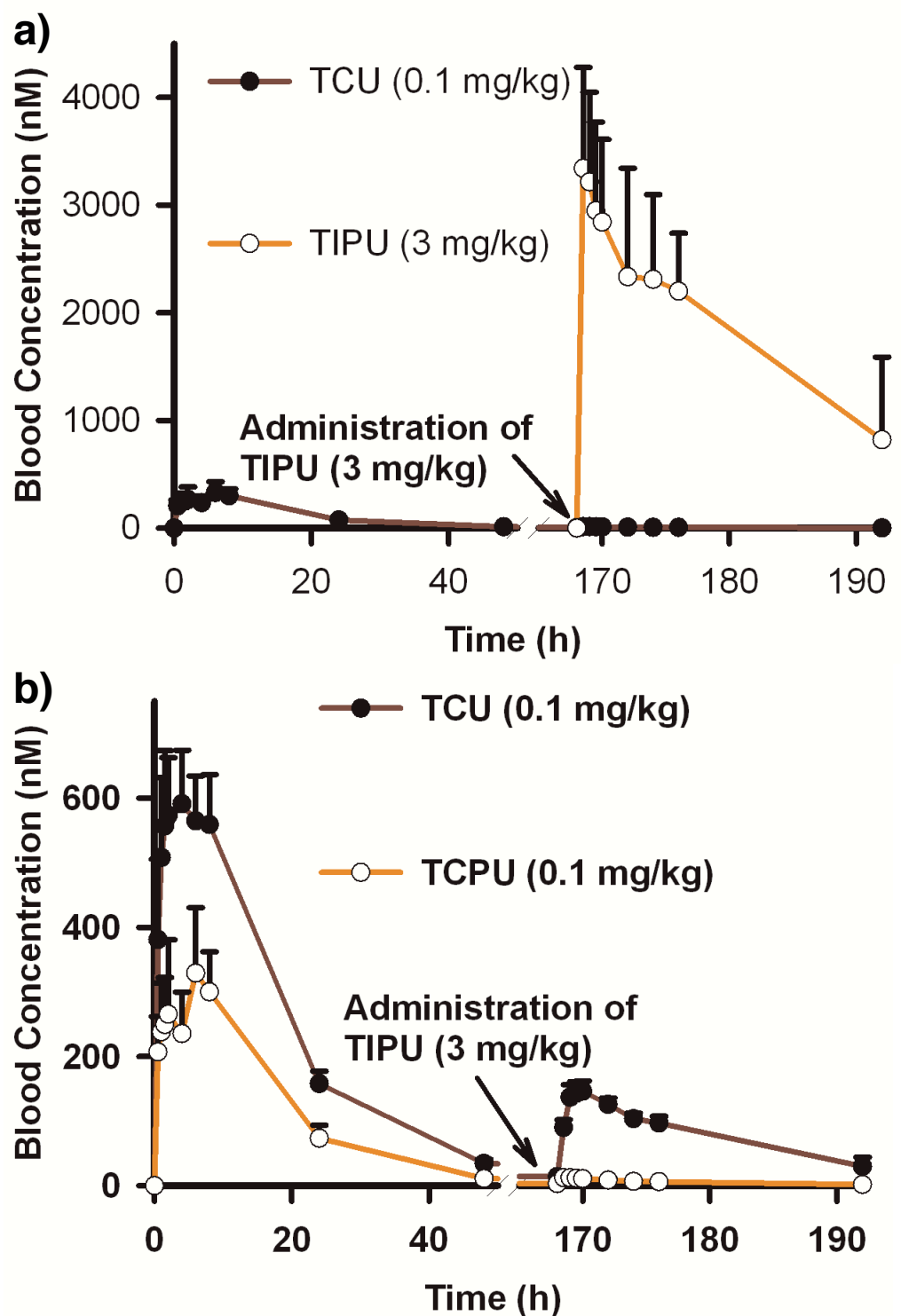
Figure S11 a) *In vivo* displacement assay of TCU and TCPU (both 0.1 mg/kg in rat with TIPU (3 mg/kg). To test the translatability of the displacement assay with different species,

we ran the displacement assay in rat. Like in the mouse study, the *in vivo* displacement assay in rat showed that the inhibitor (TCPU) with short t_R had much smaller 2nd displacement peak as compared to inhibitor (TCU) with long t_R.

b) *In vivo* displacement assay of TCU (both 0.1 mg/kg in rat with TIPU (3 mg/kg). The pharmacokinetic profile of TCU and TIPU in rat during the *in vivo* displacement assay indicate that we have enough TIPU to displace both TCU. Male rat (n=3 to 6) were treated by oral gavage (0.1 mg/kg TCU, 0.1 mg/kg TCPU or 3.0 mg/kg TIPU dissolved in PEG400, 100 to 110 µL of total injection volume depends on the weight of the rat). The data are mean ± SEM. The graphics and statistics were prepared by SigmaPlot (SysTat Software, San Jose, CA).
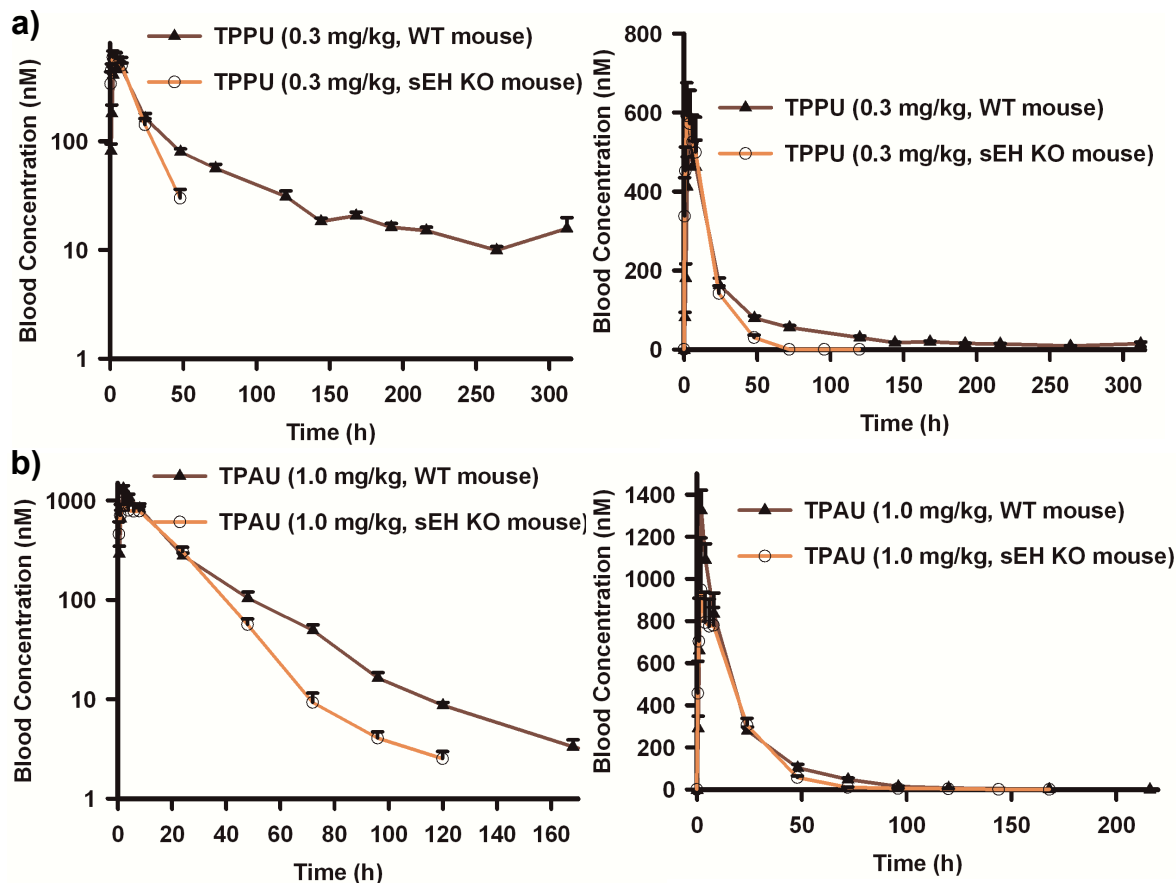
Figure S12 The pharmacokinetic (PK) profile of the sEH inhibitors are greatly affected by the t_R. The difference in PK profile between the WT and sEH KO male mice is more significant with TPPU (long t_R) than TPAU (short t_R). **a)** The full PK profile of TPPU in WT mice (solid triangle) and sEH KO mice (open circle) at 0.3 mg/kg. **b)** The full PK profile of TPAU in WT mice (solid triangle) and sEH KO mice (open circle) at 1.0 mg/kg. **Right Figure:** PK profile in log scale. **Left Figure:** PK profile in linear scale. The data are mean ± SEM. The graphics and statistics were prepared by SigmaPlot (SysTat Software, San Jose, CA).

a)

Blood Concentration (nM)

400
300
200
100
0

0   50   100   150   200

Time (h)

Administration of
TCPU at 3 mg/kg

Inset:

Blood Concentration (nM)

10000
8000
6000
4000
2000
0

0   50   100   150   200

Time (h)

- TUPS (0.3 mg/kg)
- TCPU (3.0 mg/kg)

b)

Blood Concentration (nM)

10000
1000
100
10

0   50   100   150   200

Time (h)

- TUPS (0.3 mg/kg)
- TCPU (3.0 mg/kg)

Figure S13 The PK profile of TUPS from the *in vivo* displacement assay of tested inhibitor, TUPS (0.3 mg/kg) and the displacement inhibitor, TCPU (3.0 mg/kg) in WT mice. **a)** A second peak was observed at 168h post-dosing of TUPS at 0.3 mg/kg after administration of high dose of TCPU (3 mg/kg). The linear scale of PK profile of TUPS and TCPU was inserted to show that the amount of TCPU administered is high enough to displace the sEH-bound TUPS. **b)** The PK profile (in log scale) of TUPS (0.3 mg/kg) and TCPU (3 mg/kg) throughout the *in vivo* displacement assay which indicate that we have enough TCPU to displace the TUPS bound to sEH. Male mice (n= 6) were treated by subcutaneous injection (0.3 mg/kg TUPS or 3.0 mg/kg TCPU dissolved in PEG400, 100 to 110 µL of total injection volume based on the weight of the mouse.). The data are mean ± SEM. The graphics and statistics were prepared by SigmaPlot (SysTat Software, San Jose, CA).
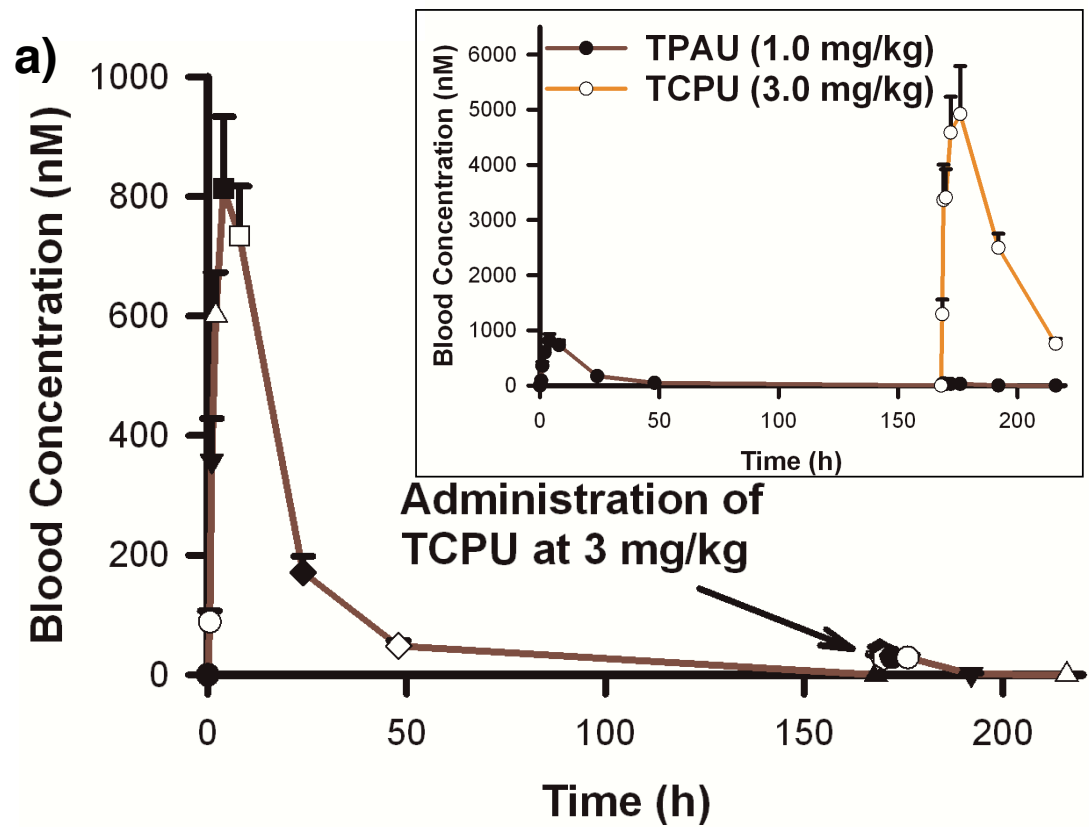
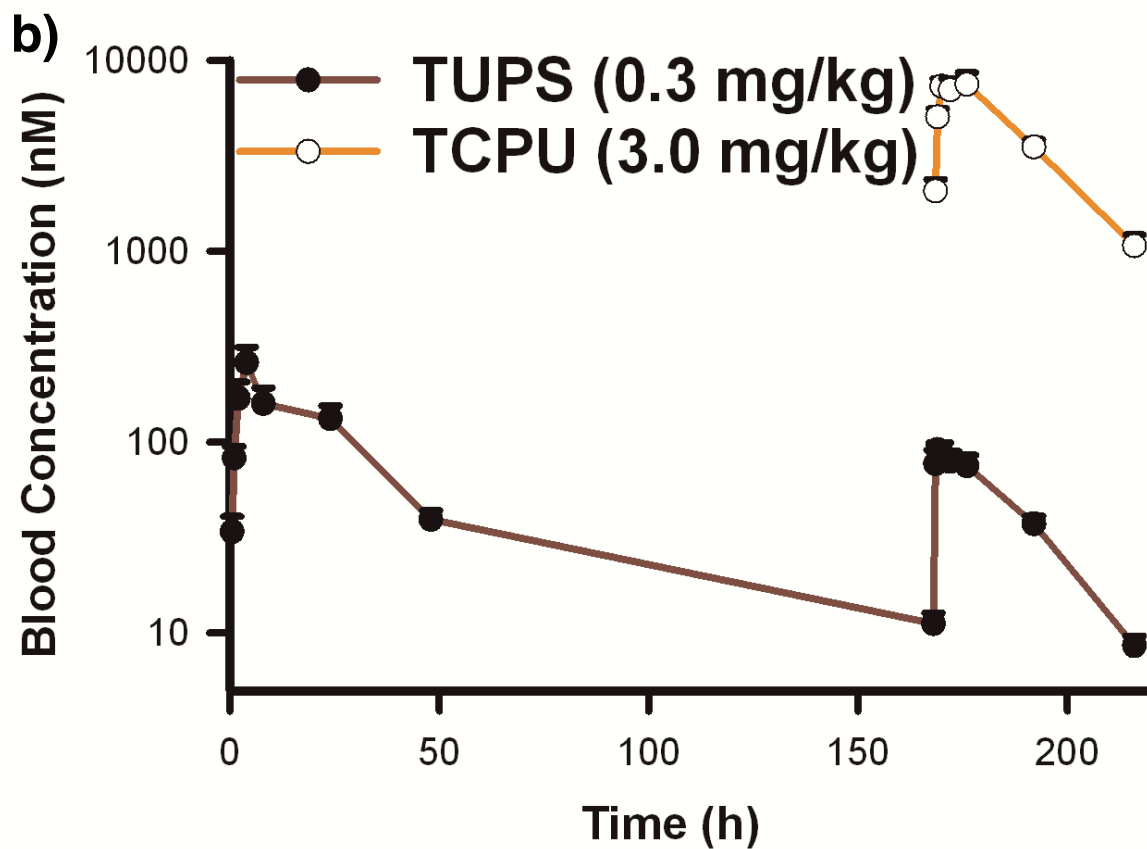*Figure S14* The PK profile of TPAU from the *in vivo* displacement assay of the tested inhibitor, TPAU (1.0 mg/kg) with TCPU (3.0 mg/kg) in WT mice. **a)** A second peak was observed at 168h post-dosing of TPAU at 1 mg/kg after administration of high dose of TCPU (3 mg/kg). The linear scale of PK profile of TPAU and TCPU was inserted to show that the amount of TCPU administered is high enough to displace the sEH-bound TPAU. **b)** The PK profile (in log scale) of TPAU (1.0 mg/kg) and TCPU (3 mg/kg) throughout the *in vivo* displacement assay which indicate that we have enough TCPU to displace the TAPU bound to sEH. Male mice (n= 6) were treated by subcutaneous injection (1.0 mg/kg TAPU or 3.0 mg/kg TCPU dissolved in PEG400, 100 to 110 μL of total injection volume depends on the weight of the mice). The data are mean ± SEM. The graphics and statistics were prepared by SigmaPlot (SysTat Software, San Jose, CA).

a)

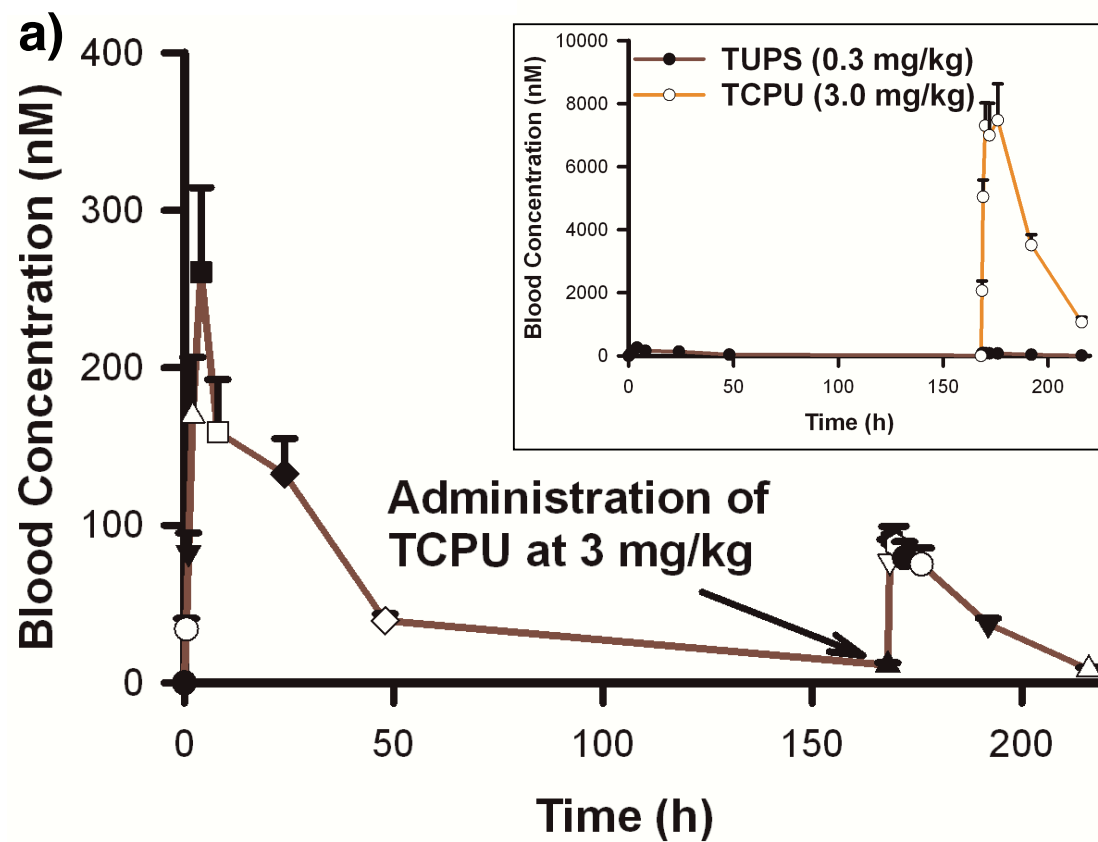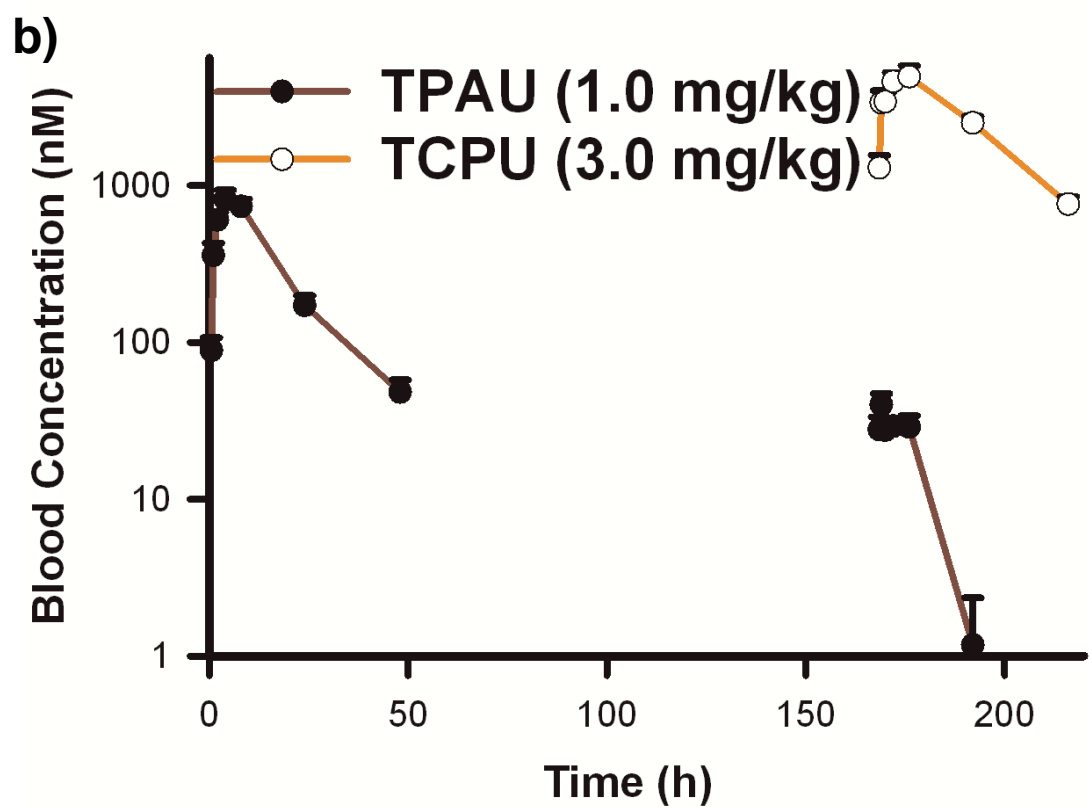**Administration of TCPU at 3 mg/kg**

Inset legend:
- TPAU (1.0 mg/kg)
- TCPU (3.0 mg/kg)

b)

- TPAU (1.0 mg/kg)
- TCPU (3.0 mg/kg)

Figure S15 The *in vivo* displacement assay of TPAU (1.0 mg/kg) as a tested inhibitor and TCPU (3.0 mg/kg) as a displacement inhibitor in sEH KO mice indicated that the 2nd displacement peak is originated from the sEH-bound inhibitor. **a)** A second peak was not observed at 168h post-dosing of TPAU at 1 mg/kg after administration of high dose of TCPU (3 mg/kg). This result indicated that there is no sEH-bound TPAU that can be displaced in the sEH KO mice. The linear scale of PK profile of TPAU and TCPU was inserted to show that the amount of TCPU administered is high enough to displace the sEH-bound TPAU if there is any. **b)** The PK profile (in log scale) of of TPAU (1.0 mg/kg) and TCPU (3 mg/kg) throughout the *in vivo* displacement assay showed that we have a high concentration of TPAU to displace the sEH-bound TPAU if there is any. Male mice (n= 6) were treated by subcutaneous injection (1.0 mg/kg TAPU or 3.0 mg/kg TCPU dissolved in PEG400, 100 to 110 µL of total injection volume based on the weight of the mice). The data are mean ± SEM. The graphics and statistics were prepared by SigmaPlot (SysTat Software, San Jose, CA).
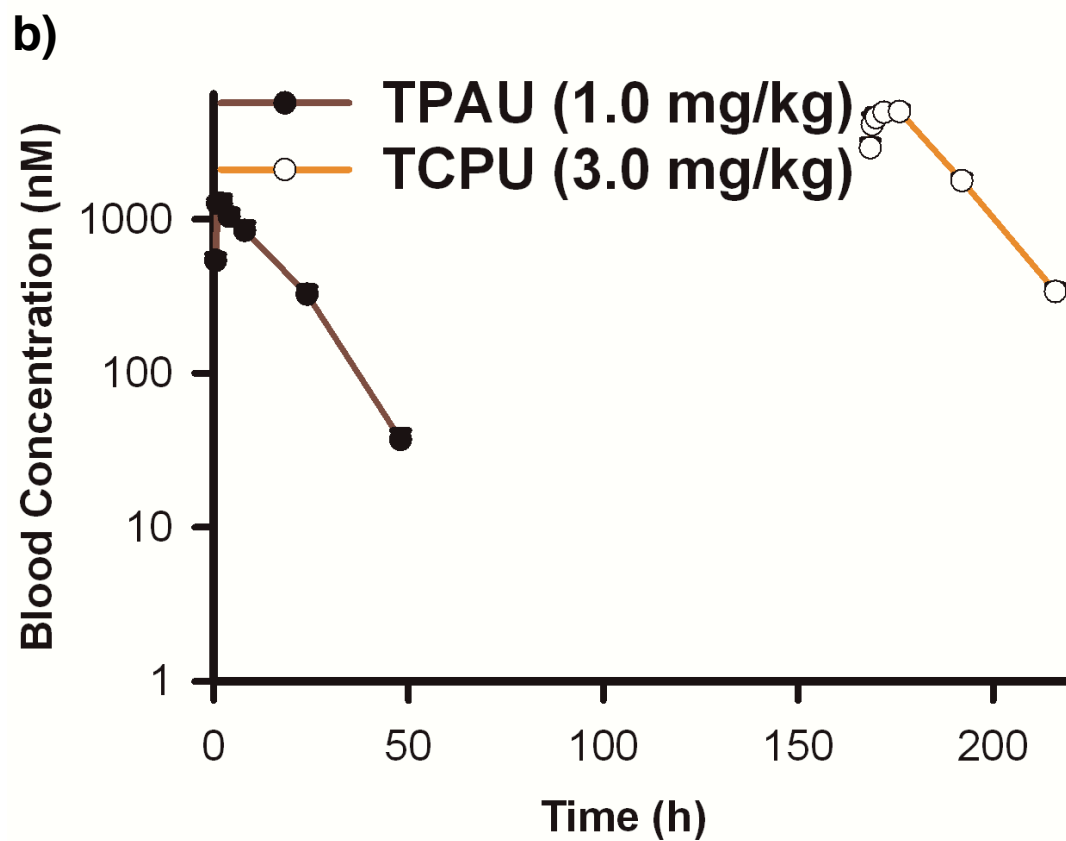
a)

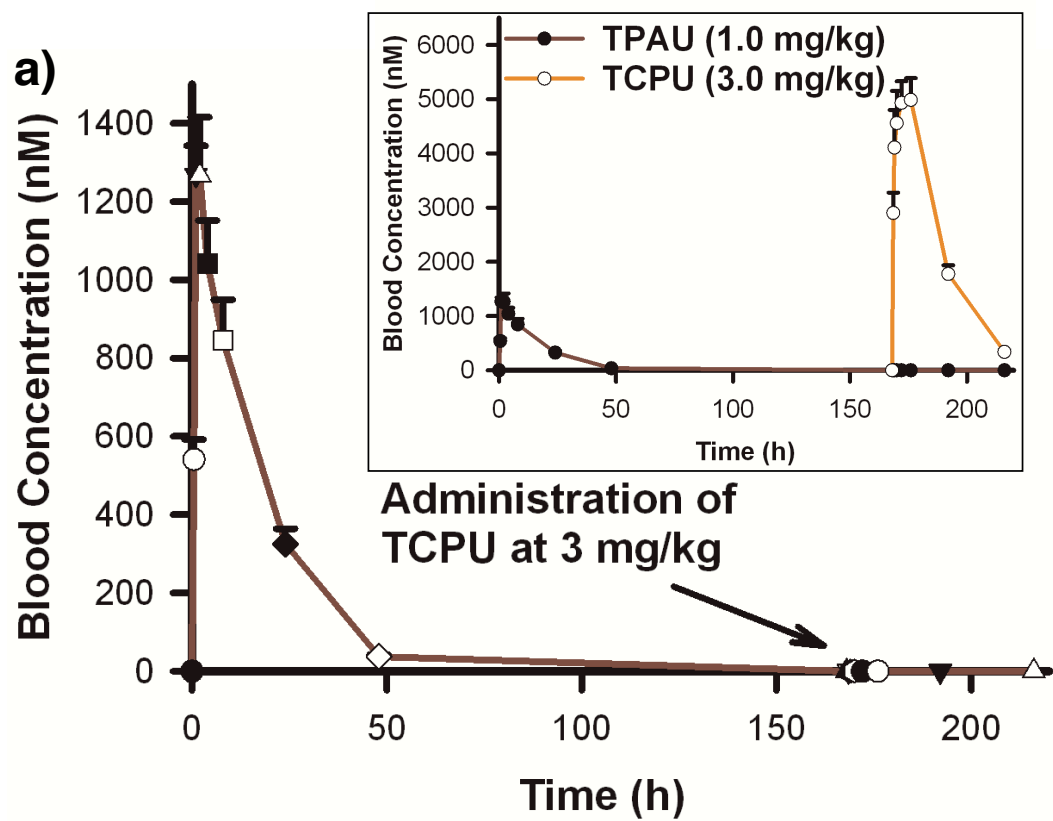**Blood Concentration (nM)** vs **Time (h)**

Inset: Blood Concentration (nM) vs Time (h), APAU(20.0 mg/kg) and TCPU (3.0 mg/kg)

Administration of TCPU at 3 mg/kg

b)

Blood Concentration (nM) vs **Time (h)**

APAU(20.0 mg/kg)
TCPU (3.0 mg/kg)

Figure S16 The *in vivo* displacement assay of metabolically liable APAU (20 mg/kg) as tested compound and TCPU (3.0 mg/kg) as a displacement compound in male mice showed that even with a metabolic liable inhibitor, a 2nd displacement peak can also be observed. The result indicates that the binding to sEH protects inhibitor from metabolism. **a)** A second peak was observed at 168h post-dosing of APAU at 20 mg/kg after administration of high dose of TCPU (3 mg/kg). These data indicate that the relatively long t_R of APAU can protect the drug from metabolism by binding to sEH. The linear scale of PK profile of APAU and TCPU was inserted to show that the amount of TCPU administered is high enough to displace the sEH-bound APAU if there is any. **b)** The PK profile (in log scale) of APAU (20 mg/kg) and TCPU (3 mg/kg) throughout the *in vivo* displacement assay shows that the TCPU level is high enough to displace any APAU bound to sEH. Male mice (n= 6) were treated by subcutaneous injection (20 mg/kg APAU or 3.0 mg/kg TCPU dissolved in PEG400, 100 to 110 µL of total injection volume). The data are mean ± SEM. The graphics and statistics were prepared by SigmaPlot (SysTat Software, San Jose, CA).
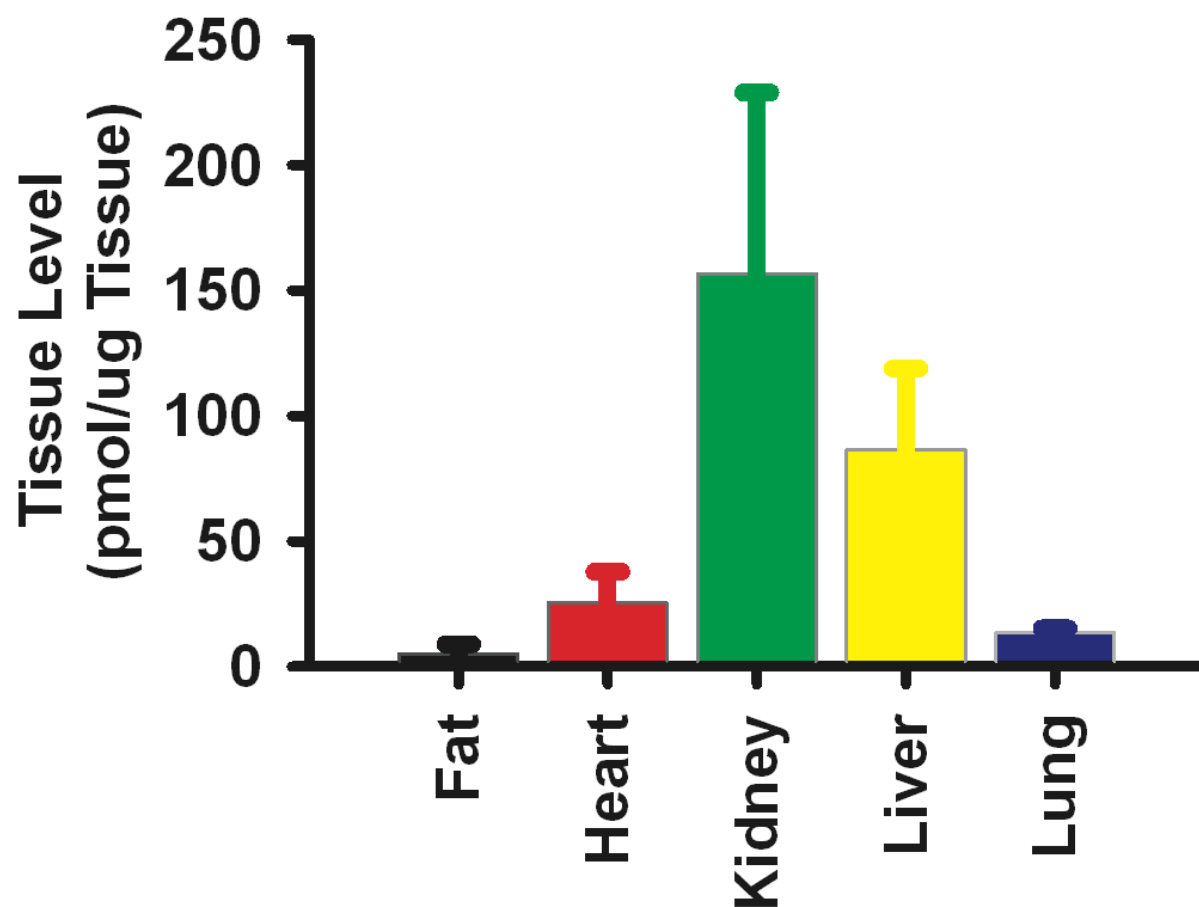
Figure S17 The tissue level of TPPU at post-dosing day 7 in different organs in the rat. It correlated well with the reported sEH level in the corresponding organ (Kidney > Liver > Heart > Lung > Fat). The data are mean ± SEM. All the graphics and statistics were prepared by SigmaPlot (SysTat Software, San Jose, CA).
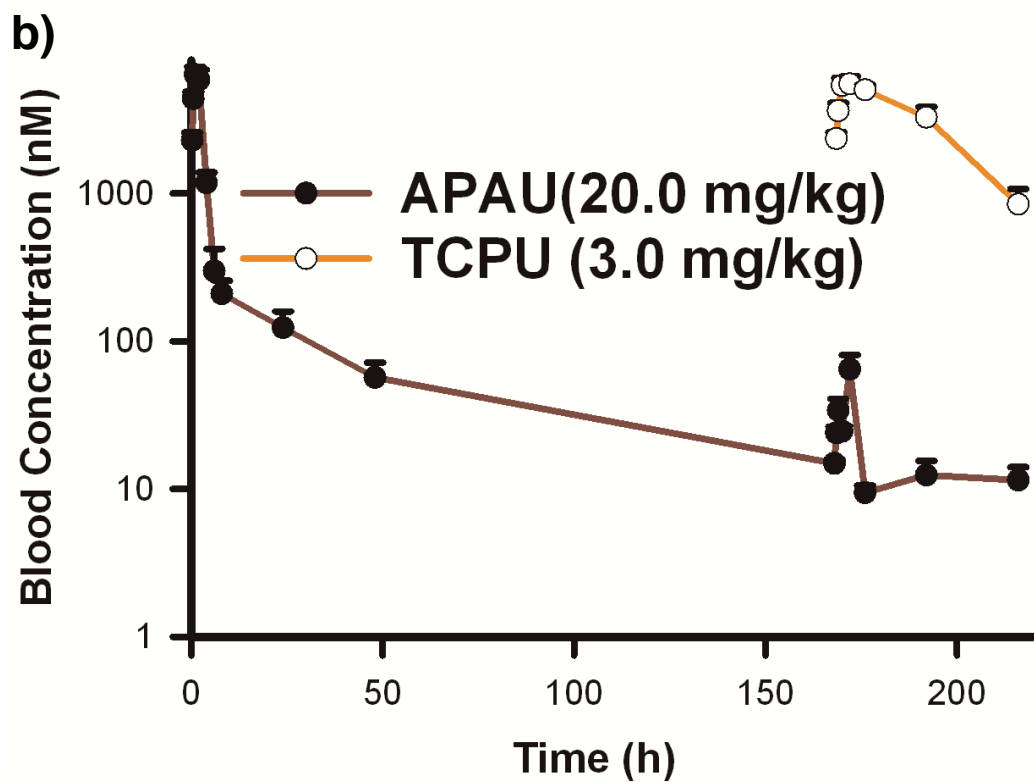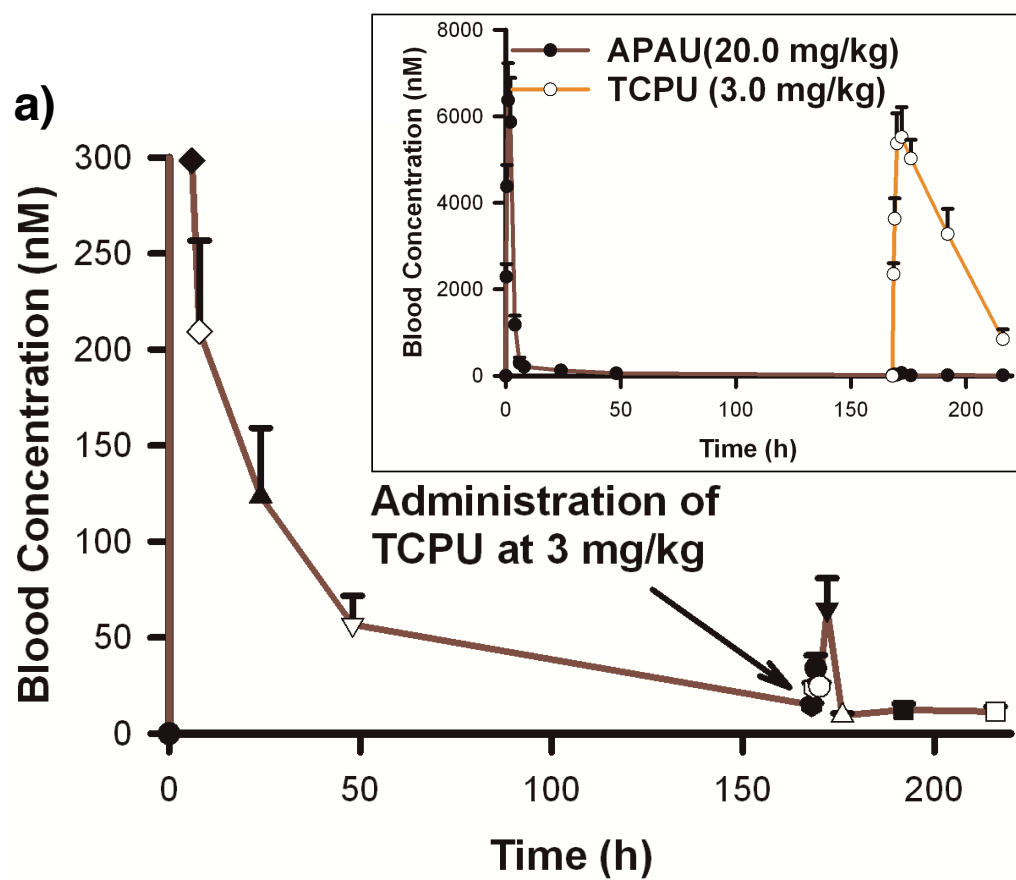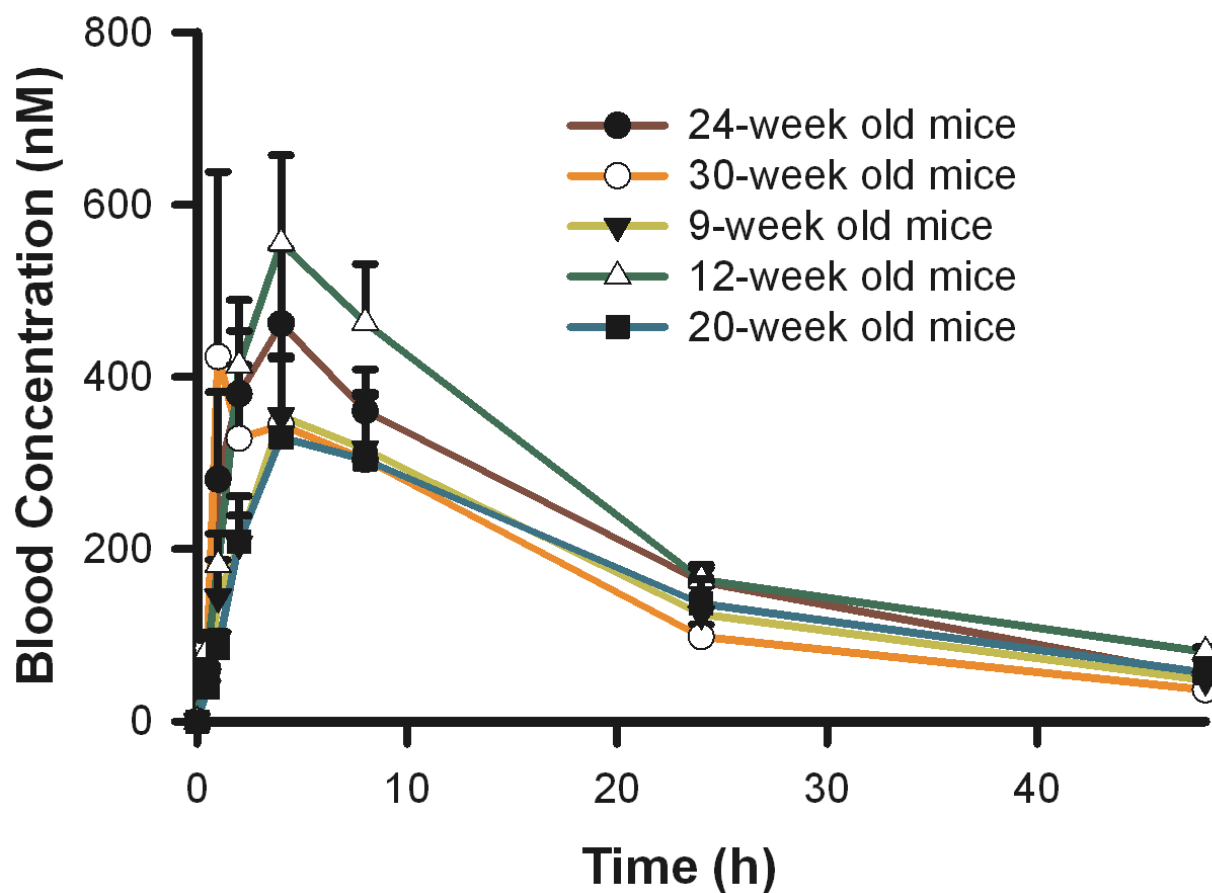
Figure S18 Comparison of PK profile of TPPU (UC1770) with different age group of mouse. The pharmacokinetic profile of TPPU (0.3 mg/kg) in male mice at different ages suggest that the age of the mouse did not affect significantly the pharmacokinetic profile of the sEH inhibitors. Gill and Hammock demonstrated a large age dependent increase in sEH activity in the liver but the changes in the target enzyme do not appear to alter overall *in vivo* pharmacokinetics profile of TPPU[1]. Mice (n=3 to 6) were treated by subcutaneous injection (0.3 mg/kg dissolved in PEG400, 100 to 110 μL of total injection volume). The data are mean ± SEM. The graphics and statistics were prepared by SigmaPlot (SysTat Software, San Jose, CA).
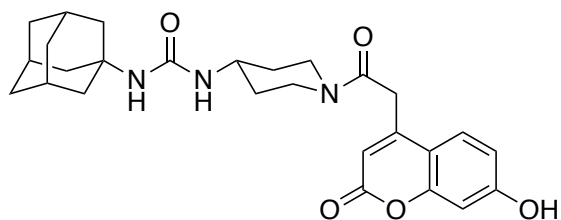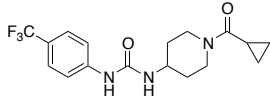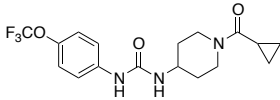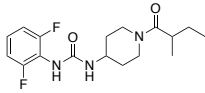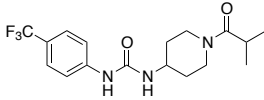
**Figure S19** The structure of ACPU, the fluorescent probe used for the fluorescent-based displacement assay to determine the K_i and k_{off} of the sEH inhibitor

2. Table S1: The *in vitro* and *in vivo* parameters of sEH inhibitors used in the rat study

Structure	[a] elogP	[a] IC_{50} (nM)	[a] $t_{1/2}$ (min⁻¹)	Dose (mg)	[b,c] PK-AUC (nM*h)	[b,c] Cmax (nM)	[b,c] PK-$T_{1/2}$ (h)	[b,c] PK-T_{max} (h)
TCU	3.18	1.4±0.1	30.7±3.0	0.1	12646	735	8.1	4.3
TCPU	3.28	9.9±0.1	15.9±1.2	0.1	6476	326	6.8	4
DFPU	N.D.	30,000	N.D.	3	8112	3234	0.7	0.4
TIPU	3.37	1.8±0.1	36.6±8.2	3	27701	2776	5.7	1.1

[a] elogP, IC_{50} and $t_{1/2}$ were reported before and IC_{50} were determined in rat sEH. [22] The results are the average of triplicates with ± SEM. [b] The mice (n=3 to 6) were treated by subcutaneous injection with inhibitor at the desired dose (0.3 to 20 mg/kg dissolved in PEG400. [c] The pharmacokinetic parameters were calculated by Winonlin based on the best-fitted models. Abbreviation: elogP stands for experimental logP.

3. Experimental Section

All the experiments presented in this manuscript are generally safe and no unexpected or unusually high safety hazards were encountered.

3.1. Pharmacokinetic (PK) protocol for an in vivo displacement assay in mouse

The animal experiments were performed based on the protocols approved by the Animal Use and Care Committee of University of California at Davis. Male C57BL/6J mice or C57BL/6J soluble epoxide hydrolase knock-out mice (n= 6, 8 to 12-week old, 24-30g were used. We did not observe different PK profile of sEH inhibitor with mice at different ages. See Figure S1). Mice were purchased from Charles River Laboratories for the PK experiments. Inhibitor (TPPU or TPAU) was dissolved in 100% PEG400 at the desired dose (0.3 or 1 mg/kg respectively) with warming at 60°C overnight to give a clear solution. The total volume of injection was limited up to 120 µL. The inhibitor was administrated through subcutaneous injection. Each group of mice in the treatment contains 3 to 6 animals. Blood (10 µL) samples were collected through tail nick at 0, 0.5, 1, 2, 4, 6, 8, 24, 48, 72, 96, 120, 144, 168, 192, 216, 264 and 312 h after administration of the inhibitor (The specific timepoint for each inhibitor will be listed in the figures). The blood sample was immediately mixed with 0.1% EDTA (w/v) in water (50 µL). All the samples were stored at -20°C until sample preparation and analysis.

3.2. Pharmacokinetic (PK) protocol for an in vivo displacement assay in rat

The animal experiments were performed based on the protocols approved by the Animal Use and Care Committee of University of California at Davis. Male Sprague-Dawley rats (n = 3 to 6, 8 to 30-week old, 24-36g were used). The animals were purchased from Charles River Laboratories for the PK experiments. Inhibitor was dissolved in 20%

PEG400 in oleic rich oil at the desired dose (0.1 mg/kg) with warming at 60˚C overnight to give a clear solution. The total volume of injection was limited and up to 1 mL was used. The inhibitor was administrated through oral gavage. At day 7 after the administration of the target inhibitor, a high dose of a second inhibitor (competitor, 3 mg/kg) dissolved in 20% PEG400 in oleic rich oil as mentioned before was administrated through oral gavage. Blood (10 µL) samples were collected through tail nick at 0, 0.5, 1, 2, 4, 6, 8, 24, 48, 168, 168.5, 169, 170, 172, 174, 176 and 192h after administration of the inhibitor (The specific timepoint for each inhibitor will be listed in the figures). The blood sample was immediately mixed with 0.1% EDTA (w/v) in water (50 µL). All the samples were stored at -20˚C until sample preparation and analysis.

3.3. Analysis of the sEH inhibitors in blood samples

The blood samples were prepared according to the published method by Liu *et al.* The concentration of the inhibitors was determined based on the previous published method by Lee *et al* unless specified. [2] The table below summarized the optimized conditions for monitoring sEH inhibitors by multiple reaction-monitoring mode (MRM) on the 4000 Q-TRAP triple quadrupole mass spectrometer (Applied Biosystems, Forster City, CA)

3.3.1. Table S2) Optimized conditions for monitoring parent sEH inhibitors by MRM on 4000 Q-TRAP triple quadrupole mass spectrometer

Inhibitor	Transition (Q1-Q3)	Declustering Potential (V)	Entrance Potential (V)	Collision Energy (V)	Collision Cell Exit Potential (V)	LOD (nM)

TPPU	358.2-175.9	-125	-10	-22	-11	≤0.49
TAPU	327.9-159.8	-100	-10	-24	-11	≤0.49
TIPU	356.2-159.9	-110	-10	-26	-1	≤0.49
TPCU	354.2-159.9	-110	-10	-22	-7	≤0.49
CUDA (Internal Standard)	339.1-214.3	-65	-10	-32	-4	≤0.49
TCPU	370.2-176.1	-90	-10	-22	-9	≤0.49
TPAU	343.9-176.0	-105	-10	-22	-7	≤0.49
TUPS	380.0-176.0	-95	-10	-20	-5	≤0.49
mTPPU	358.1-175.9	-90	-10	-22	-7	≤0.49

3.3.2. Table S3) Optimized LC conditions for the analysis of sEH inhibitor.

Step	Total Time (min)	Flow Rate (μl/min)	A (%)	B (%)
0	0	300	65.0	35.0
1	3.5	300	20.0	80.0
2	3.6	400	2.0	98.0
3	4.0	400	2.0	98.0
4	4.1	400	65.0	35.0
5	5.0	400	65.0	35.0

Buffer A: 0.1% acetic acid in milli-Q purified water; Buffer B: 0.1% acetic acid in acetonitrile (HPLC grade)

3.4. PK analysis

The PK parameters of individual mice were calculated by fitting the time course curve of blood concentration data to a non-compartmental analysis with the WinNonlin software (Pharsight, Mountain View, CA). Parameters estimated include time of maximum concentration (T_{max}), maximum concentration (C_{max}), half life (t1/2), and area under the concentration–time curve to terminal time (AUC_t). AUC was calculated by the linear/log trapezoidal rule.

3.5. Chemical Synthesis

All chemicals and solvents purchased from commercial suppliers were used directly without purification. All syntheses were carried out in dry argon atmosphere with oven-dried reaction vessels unless specified. Reactions were monitored by thin-layer

chromatography (TLC, on silica gel, 60Å, glass slides, fluorescent indicator coated, spots were visible under UV-light (254 nm) or stained with potassium permanganate solution). Column chromatography was conducted on silica gel.

^{1}H-NMR spectra were performed on a Varian QE-300 spectrometer with deuterated chloroform (CDCl$_3$, ∂ = 7.24 ppm) or deuterated dimethyl sulfoxide (DMSO-d_6, ∂ = 2.50 ppm).

The purity of the inhibitors reported in this manuscript was determined either by 1) HPLC-UV using Shimadzu Prominence HPLC coupled with Phenomenex Kinetex C18 reverse phase column (4.6 x 250 mm, 5 μm, 100Å) using 230 nm for detection with linear gradient from 5% water in acetonitrile to 100% acetonitrile in 30 min; and by 2) ^{1}H-NMR. The lowest purity was reported. The inhibitors were dissolved in ethanol at 100 μM and 10 μL was injected to HPLC. The purity was estimated based on the inhibitor's peak area at 230 nm relative to the total peak area at 230 nm using HPLC-UV. The syntheses of most compounds, have been reported elsewhere. [2-4]

3.5.1. The synthesis of 1-(1-((λ^1-methyl)(λ^1-oxidaneyl)boraneyl)piperidin-4-yl)-3-(2,6-difluorophenyl)urea (*1*)
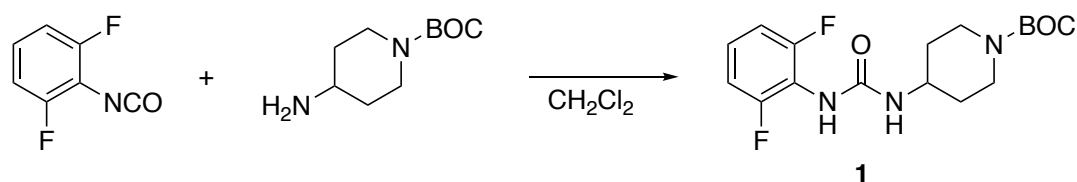
2,6-Difluorophenyl isocyanate (270 mg, 1.7 mmol) and 1-Boc-4-aminopiperidine (393 mg, 2.0 mmol) was dissolved in DCM (8 mL). Reaction was stirred for 18 hours and was

quenched by addition of 1M HCl . The organic layer was collected and the aqueous layer was extracted three times with EtOAc. The resulting organic solution was concentrated *in vacuo* and purified by flash chromatography (EtOAc/Hexane 3:7), giving **1** (572 mg, 93% yield).

^{1}H NMR (300 MHz, DMSO-d6) δ 7.74 (s, 1H), 7.29-7.16 (m, 1H), 7.13-7.02 (m, 2H), 6.38 (d, J = 7.8 Hz, 1H), 3.82 (d, J = 12.9 Hz, 2H), 3.66-3.53 (m, 1H), 2.98-2.78 (m, 2H), 1.85-1.72 (m, 2H), 1.40 (s, 9H), 1.34-1.18 (m, 2H).

Melting Point = 204.8-207.9 (205.3) °C.

HRMS expected [M-H]$^-$ = 355.1707 observed [M-H]$^-$ = 355.1151.

3.5.2. The synthesis of 1-(2,6-difluorophenyl)-3-(piperidin-4-yl)urea (*2*)
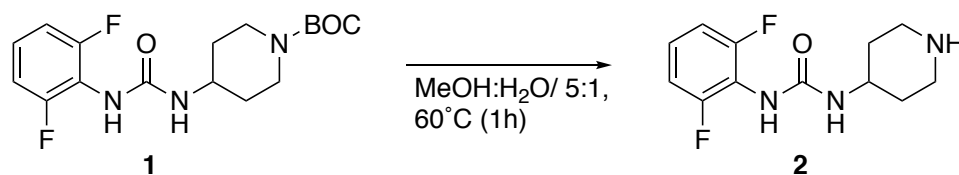
To remove the Boc protecting group, **1** (286 mg, 0.8 mmol) was dissolved in a 5:1 MeOH:HCl solution and stirred at 60° Celsius for 1 hour. The resulting solution was concentrated *in vacuo*. The resulting solution was extracted twice with hexane. NaOH was then added to the solution until it turned basic. The product was then extracted with EtOAc and dried over MgSO$_4$. Evaporation of the product led to **2** (96 mg g, 47% yield).
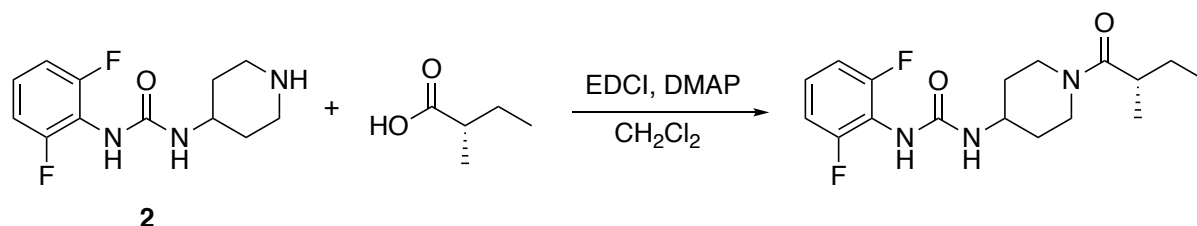
^{1}H NMR (300 MHz, DMSO-d6) δ 7.72 (s, 1H), 7.29-7.16 (m, 1H), 7.13-7.03 (m, 2H), 6.35

(d, J = 7.5 Hz, 1H), 3.54-3.39 (m, 1H), 2.98-2.85 (m, 2H), 2.58-2.42 (m, 2H), 1.81-1.68

(m, 2H), 1.34-1.17 (m, 2H).

Melting Point = 175.3-176.5 (175.6) °C.

HRMS expected $[M+H]^+$ = 255.1183 observed $[M+H]^+$ = 255.0312.

3.5.3. *The synthesis of (S)-1-(2,6-difluorophenyl)-3-(1-(2-methylbutanoyl)piperidin-4-yl)urea*

(S)-(+)-2-Methylbutyric acid (30 mg, 0.29 mmol), **2** (75 mg, 0.29 mmol), dimethylaminopyridine (DMAP, 30 mg, 0.25 mmol) and EDCI (60 mg, 0.37 mmol) were dissolved in DCM (5 mL) and allowed to stir overnight. The reaction was removed from stirring and extracted once with 0.2 M HCl. The organic layer was dried over MgSO4 and concentrated *in vacuo*. The product was purified by flash chromatography on 1:1 Hex:EtOAc yielding **1** (40 mg, 41%, purity %).

^{1}H NMR (300 MHz, DMSO-d6) δ 7.73 (d, *J* = 6.9 Hz, 1H), 7.29-7.17 (m, 1H), 7.14-7.03 (m, 2H), 6.41 (t, *J* = 6.6 Hz, 1H), 4.30-4.17 (broad, 1H), 3.88 (d, *J* = 13.8 Hz, 1H), 3.74-3.60 (m, 1H), 3.21-3.05 (m, 1H), 2.81-2.63 (m, 2H), 1.94-1.72 (m, 2H), 1.62-1.47 (broad, 1H), 1.37-1.12 (m, 3H), 0.97 (t, *J* = 6.3 Hz, 3H), 0.81 (q, *J* = 7.2 Hz, 3H).

Melting Point = 144.7-146.7 (145.3) °C.

HRMS expected $[M+H]^+$ = 339.1758 observed $[M+H]^+$ = 339.1189.

Elemental Analysis: C = 60.24%, H = 6.92%, N = 12.26%.

3.5.4. *The synthesis of 1-(1-propionylpiperidin-4-yl)-3-(2-(trifluoromethoxy)phenyl)urea*
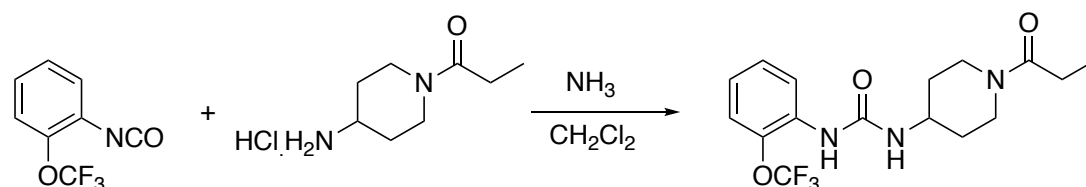
2-(trifluoromethoxy)phenyl isocyanate (100 mg, 492.3 μmol) was added to a solution of trimethylamine (52.2 mg, 516.9 μmol) and 1-propionyl-4-piperidiamine hydrochloride salt (99.6 mg, 516.9 μmol) dissolved in dichloromethane (50 mL). The reaction was quenched with the addition of hydrochloric acid solution (1M, 50 mL). The organic layer was collected was further washed by hydrochloric acid solution (1M, 50 mL). The crude was concentrated under vacuum and the product was purified by re-crystallization with methanol and water resulting 55% yield. (97.3 mg, 271 μmol, 98% purity)

^{1}H NMR (500 MHz, DMSO-d6) δ 8.26 (d, *J* = 10 Hz, 1H), 8.12 (s, 1H), 7.24-7.33 (m, 2H), 6.9-7.1 (m, 2H), 4.16 (d, *J* = 15Hz, 1H), 3.76 (d, *J* = 15 Hz, 1H), 3.72-3.65 (m, 1H), 3.15 (t, *J* = 10 Hz, 1H), 2.82 (t, *J* = 10 Hz, 1H), 2.33 (q, *J* = 10 Hz, 2H), 1.75-1.95 (m, 2H), 1.10-1.35 (m, 2H), 0.98 (t, *J* = 7.5 Hz, 3H).

3.6. Biochemistry

Expression and purification of recombinant sEH followed the published procedure. [5] The purity of the mouse sEH was higher than 95% as reported before. [3]

IC_{50} determination for sEH inhibitors

The IC_{50} of sEH inhibitors were determined by published method. [6-7] IC_{50}s of the inhibitor, the concentration of the inhibitor that inhibits 50% of the enzyme activity, were determined with the linear regression of at least 5 datum points with minimum of two points in the linear region of the curve on either side of the IC_{50}.

3.6.1. Measurement of K_i using FRET displacement assay

The FRET displacement assay was carried out based on the published procedure. [2-3] Briefly, to avoid leaching of fluorescence impurities from the plastic container and non-specific binding to sEH inhibitors, the inhibitors were dissolved in DMSO and stored in glass vials.

1) Preparation of the FRET displacement assay

The 96 well-plate for the assay was pre-incubated in sodium phosphate buffer (PB, 100 mM sodium phosphate, 0.01% gelatin, pH 7.4) for at least 8h in order to pre-coated the 96 well-plate with gelatin to prevent sEH inhibitor and sEH from being absorbed by the plastic. The buffer was discarded, and the plate was ready to be used

2) High-throughput K_i determination

The sEH was further diluted to 20 nM by PB (100 mM sodium phosphate, pH 7.4). ACPU (one equivalent to sEH in the solution, 10 mM stock in ethanol, Figure S18) was added to the sEH solution. The sEH:ACPU solution was mixed thoroughly and was incubated for 2h at rt in the dark. The sEH:ACPU mixture (20 nM each, 100 mM sodium phosphate, pH 7.4, 150 µL) was added to each well. The fluorescent baseline (F_0) ($\lambda_{excitation}$: 280 nm, $\lambda_{emission}$: 450 nm) of the samples was measured after z position and gain were fully optimized by the fluorometer. The z position and gain were noted and were used as the setting for measuring the sample fluorescent after the inhibitor was added. DMSO which

is the solvent used for the inhibitor has been reported to quench the fluorescence. Therefore, 1% DMSO in PB was served as a control (F_{DMSO}). The desired amount of inhibitor was added to the first well. The desired concentration of the inhibitor in the first well was two-time of the concentration where 100% of the sEH was bound to the inhibitor. The first well was then diluted by 2-fold with the PB buffer and was then futher diluted 2-fold across the rest of the row of well (12 wells in total). 12-datum points which corresponding to 12 different concentration of the inhibitor is generally sufficient data to caculate an accurate K_i of the inhibitors. The samples were then incubated at 30°C for 1.5h. The fluorescence ($\lambda_{excitation}$ = 280 nm, $\lambda_{emission}$ = 450 nm) of the samples were then measured using the z position and gain setting that was previously optimized. The obtained fluorescence data were transformed as below and the transformed data were used to determine the K_i of the inhibitor based on the published procedure without modification. [2-3] The data manipulation and K_i calculation were based on the original paper by Wang *et al.* with modifications suggested by Roehrl *et al.*[8-9]

Initiated fluorescence = $F_{DMSO\ (well\ X)}$ / $F_{0\ (well\ X)}$
Saturated fluorescence = $F_{at\ the\ saturated\ concentration\ (well\ X)}$ / $F_{0\ (at\ well\ X)}$
Observed fluorescence = $F_{(well\ X)}$ / $F_{0\ (well\ X)}$

3.6.2. k_{off} measurement procedure

The k_{off} was determined as described before. [2-3] Briefly, the sEH (8 µM) was pre-incubated with the inhibitor (8.8 µM, 100 mM PB buffer, pH 7.4) for 2h at rt. The sEH:inhibitor complex was then diluted by 40-fold with ACPU solution (20 µM, 100 mM Sodium phosphate buffer, pH 7.4). The fluorescence ($\lambda_{excitation}$ = 280 nm, $\lambda_{emission}$ = 450 nm) was monitored immediately for 5100s every 30s. The fluorescence ($\lambda_{emission}$ = 450 nm) data

was plotted against time (s). The resulting curve was fitted to single exponential growth and the relative k_{off} was calculated.

3.6.3. Determination of plasma to blood ratio of sEH inhibitor

The mouse blood was a generous gift from Dr. Liby. Briefly, selected sEH inhibitor (0.5 mM, 0.3 μL, DMSO stock) was added to heparinized freshly connected mouse blood (300 μL). The blood sample was incubated at 37°C and shaken at 500 rpm for 1h. A sample from whole blood (50 μL) was collected and the rest of the whole blood was centrifuged at 1,500 g in microfuge at 4 °C for 10 min. The plasma sample (50 μL) was collected. An internal standard (1 μL, an sEH inhibitor which is structurally similar with similar log P as the tested inhibitor) was added to both plasma and whole blood sample and was mixed in the shaker for 1 min at 1000 rpm. Both samples were then extracted with ethyl acetate (200 μL) twice. The collected organic layers were combined and was evaporated under vacuum. The dried samples were reconstituted by adding 50 μL of external standard (50 μL, 100 nM CUDA (a sEH inhibitor). The samples were then storage at -20 °C until LC/MSMS analysis.

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