Supporting Information

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SI Materials and Methods

Cell Culture and Materials. Cancer cells, including human prostate cancer (PC-3), mouse breast cancer (Met-1), mouse Lewis lung carcinoma (LLC), and mouse melanoma B16F10 from the American Type Culture Collection, were cultured in MEM-α medium supplemented with 10% (vol/vol) FBS (Invitrogen) and 1% antibiotics in a 37 °C cell culture incubator under a 5% CO_2 atmosphere. Human umbilical vein endothelial cells (HUVECs, Clonetics) from Lonza were cultured in EBM-2 medium with supplements according to the manufacturer's instructions. Assays with HUVECs were conducted with cells from passages 2–6.

A cyclooxygenase (COX)-2/soluble epoxide hydrolase (sEH) dual inhibitor 4-(5-phenyl-3-{3-[3-(4-trifluoromethyl-phenyl)-ureido]-propyl}-pyrazol-1-yl)-benzenesulfonamide (PTUPB) and other COX-2/sEH dual inhibitors were synthesized as described (1). Two sEH inhibitors (sEHIs) of quite different structures but similar potency on the affinity-purified murine recombinant sEH were used in the study to test the hypothesis that the sEHI effects were due to sEH inhibition and were not just structure-dependent. The two sEHIs are *trans*-4-[4-(3-adamantan-1-yl-ureido)-cyclohexyloxy]-benzoic acid (*t*-AUCB) (2) and 1-trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl)urea (TPPU) (3). The structures of the compounds were supported by NMR, IR, and MS, and high purity was indicated by TLC, HPLC-MS, and the melting point.

Cell Proliferation Assay. The cells were plated into 96-well plates at a cell density of 1–1.5 × 10^3 cells per well in 100 μL of complete medium. After 24 h, the medium was replaced with fresh medium containing test compounds. After 72 h of treatment, cell viability was assessed by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma–Aldrich) or BrdU assay (Millipore). PTUPB was also subjected to a National Cancer Institute-60 human cancer cell line screening, in which the cancer cells were treated with PTUPB for 48 h.

Tube Formation Assay. The 96-well plates were coated with 35 μL per well of growth factor-reduced Matrigel (BD Biosciences) and solidified at 37 °C for 30 min; 1.5–2 × 10^4 HUVECs in 100 μL of basal medium were then added to each well. After 6 h of treatment, microscope images were recorded (4).

Mouse Aortic Ring Assay. The aortas of C57BL/6 mice were dissected, washed with serum-free F-12K medium five to six times, and then cut to ∼1-mm length rings. The aortic rings were placed in 48-well plates that were precoated with 100 μL per well of growth factor-reduced Matrigel and incubated at 37 °C for 10 min; another 100 μL of Matrigel was then added into each well and solidified at 37 °C for 30 min. Complete endothelial culture medium (500 μL) containing test compounds was added into each well. After 4–8 d of treatment, microscopic images were recorded (5).

Oris Cell Migration Assay. HUVECs were plated into 96-well plates (Platypus Technologies) with stoppers at a cell density of 2 × 10^4 cells per well in 100 μL of complete endothelial medium. After 24 h, the stoppers were removed, the medium was decanted, and the wells were washed using PBS buffer and treated with test compounds in complete medium. After 24 h of treatment, the cells were stained with Calcein AM (Caymen Chemicals) and fluorescence microscope images were recorded.

Boyden Chamber Cell Migration Assay. HUVECs or LLC cancer cells (5 × 10^4 cells per well) were seeded onto the upper chamber of cell culture inserts for a 24-well plate containing membrane with 8-μm pores (BD Biosciences) in 400 μL of serum-free medium, and the bottom chamber was filled with 500 μL of complete medium as chemoattractant. The test compounds were added into both the top and bottom chambers. After 18–24 h of incubation, the inserts were washed with PBS and fixed with 5% glutaraldehyde for 30 min, and the unmigrated cells were removed with cotton swabs. The migrated cells were stained with crystal violet, and microscope images were recorded and migrated cells were counted using ImageJ (National Institutes of Health) software (6).

Gelatin Zymography. HUVECs were seeded in six-well plates in complete endothelial medium and allowed to attach overnight, and the cells were then treated with test compounds for 24–72 h. Matrix metalloproteinase activities in the cell culture medium were analyzed by zymography using Novex 10% Zymogram (Gelatin) Gel (Invitrogen).

Immunoblotting. For cell cycle analysis, HUVECs in six-well plates were treated with PTUPB for 24 h. For VEGF receptor 2 (VEGFR2) signaling, HUVECs were serum-starved in basal medium for 24 h and then treated with 50 ng/mL VEGF and PTUPB for 10 min. The medium was decanted and washed with cold PBS buffer; the cells were then lysed and the cell lysates were resolved using SDS/PAGE and transferred onto a nitrocellulose membrane. The membranes were blocked in 5% nonfat dry milk for 1 h at room temperature and probed with mouse monoclonal anti-cyclin–dependent kinase 4 (CDK4) and anti-cyclin–dependent kinase 6 (CDK6) antibodies (Cell Signaling Technology), mouse monoclonal anti-p53 antibody (Santa Cruz Biotechnology Inc.), rabbit polyclonal anti-cyclin D1 antibody (Cell Signaling Technology), anti-p–VEGFR2 antibodies (Cell Signaling Technology), and mouse monoclonal anti–β-actin antibody (Sigma–Aldrich). The membranes were then probed with HRP-tagged secondary antibodies. The secondary antibodies on the blot were detected by an ECL Plus Western Blotting Detection Reagent (GE Healthcare).

Cell Cycle Analysis. HUVECs were treated with PTUPB for 24 h in flasks. After treatment, the cells were collected, washed with cold PBS, and fixed in 70% ethanol at −20 °C for at least 3 h. The cells were then washed with PBS and stained with propidium iodide. After staining, the cells were processed for cell cycle analysis using Muse Cell Analyzer from Millipore following the manufacturer's instructions.

Matrigel Plug Angiogenesis Assay. All procedures and animal care were performed in accordance with the protocols approved by the Institutional Animal Care and Use Committee of the University of California, Davis. A total of 0.5 mL of growth factor-reduced Matrigel was mixed with 100 ng of mouse VEGF 164 (R&D Systems) and 20 units of heparin (APP Pharmaceuticals), with or without PTUPB. The gel was then s.c. injected into C57BL/6 mice in the abdominal area. After 4 d, the animals were euthanized to dissect the implanted Matrigel plugs. The gel plugs were weighed, homogenized in 1 mL of PBS buffer, and centrifuged, and the content of hemoglobin in the supernatant was analyzed using Drabkin's reagent (Sigma–Aldrich) and normalized to the gel weights. Endothelial cell invasion into the gel plugs were characterized by the immunohistochemistry of CD31 staining (7).

Lipidomics. For plasma lipid mediator extraction, Waters Oasis solid phase extraction (SPE) cartridges were prewashed, and 250 μL of plasma with surrogate solution was loaded onto the SPE column and washed with 95:5 (vol/vol) water/methanol with 0.1% acetic acid. The analytes were eluted with methanol and ethyl acetate, dried, and then reconstituted in methanol for LC-tandem MS (MS/MS) analysis. For tumor lipid mediator extraction, ~100 mg of tumor tissues was mixed with the antioxidant solution (0.2 mg/mL butylated hydroxytoluene and 0.2 mg/mL triphenylphosphine in methanol), the surrogate solution, and 400 μL of extract solution (0.1% acetic acid with 0.2 mg/mL butylated hydroxytoluene in a methanol solution), and then homogenized; the resulting homogenates were kept at −80 ° overnight. After centrifugation of the homogenates, the pellets were washed with methanol with 0.1% butylated hydroxytoluene and 0.1% acetic acid, and then combined with the supernatant. The combined solutions were extracted using SPE columns as described above. The LC-MS/MS analysis was carried out on an Agilent 1200SL liquid chromatographic system coupled to a 4000 QTRAP MS/MS instrument (AB Sciex) as described (8).

1. Hwang SH, et al. (2011) Synthesis and structure-activity relationship studies of urea-containing pyrazoles as dual inhibitors of cyclooxygenase-2 and soluble epoxide hydrolase. *J Med Chem* 54(8):3037–3050.
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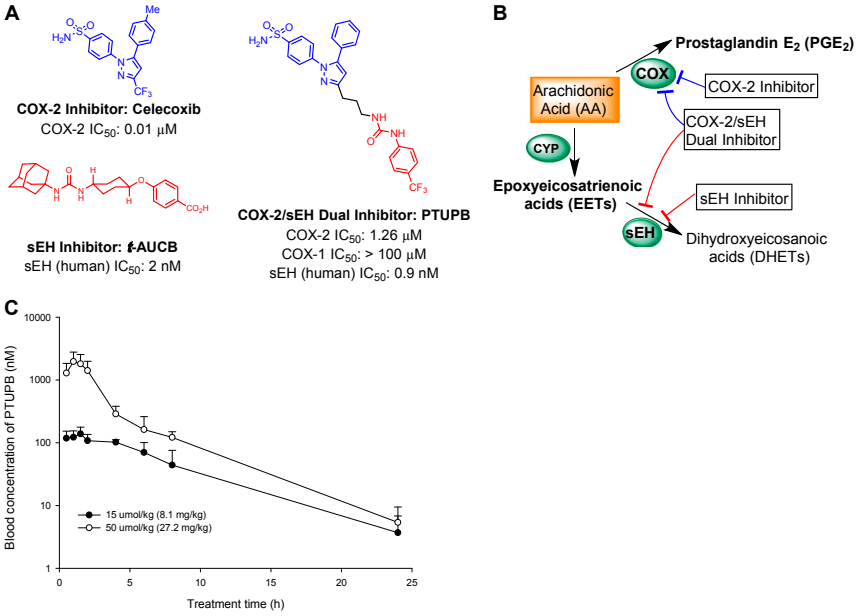


Fig. S1. (*A*) Chemical structures of the COX-2–selective inhibitor celecoxib, the sEHI *t*-AUCB, and the COX-2/sEH dual inhibitor PTUPB. (*B*) Simplified scheme of arachidonic acid cascade; the COX-2/sEH dual inhibitor inhibits both COX-2 and sEH pathways. (*C*) Time course of blood concentration of PTUPB following oral administration to mice. Data represent mean ± SD ($n = 3$). At a dose around 30 mg/kg, the plasma concentration of PTUPB reached above its IC_{50} value for COX-2.

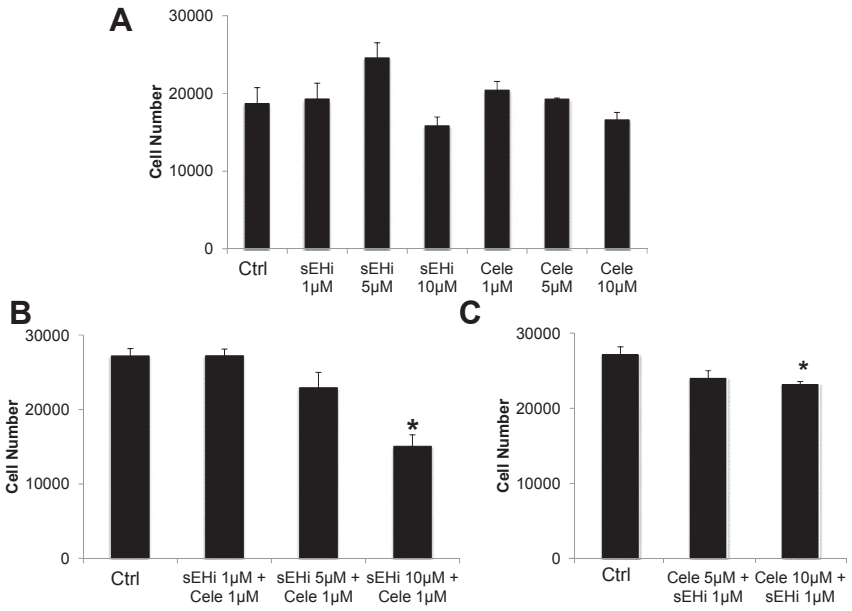


Fig. S2. (*A*) sEHI *t*-AUCB or celecoxib (Cele) alone at 1–10 μM had no effect on the proliferation of bovine capillary endothelial cells (BCEs). (*B*) Combination of celecoxib (1 μM) with varied doses of *t*-AUCB (1–10 μM) on BCE proliferation. Coadministration of *t*-AUCB (10 μM) and celecoxib (1 μM) significantly inhibits the proliferation of BCEs. (*C*) Combination of *t*-AUCB (1 μM) with varied doses of celecoxib (5–10 μM) on BCE proliferation. To show the potential synergistic activity, we used BCE cells and induced their proliferation with basic fibroblast growth factor (bFGF), a potent mitogen for BCE cells. Proliferation of BCE cells is determined by comparing cells exposed to an angiogenic stimulus (bFGF) with those exposed to bFGF and sEHI (*t*-AUCB) and/or celecoxib, relative to unstimulated cells (low serum), in a 72-h proliferation assay. The results are expressed as mean ± SD. $*P < 0.05$. Ctrl, control.

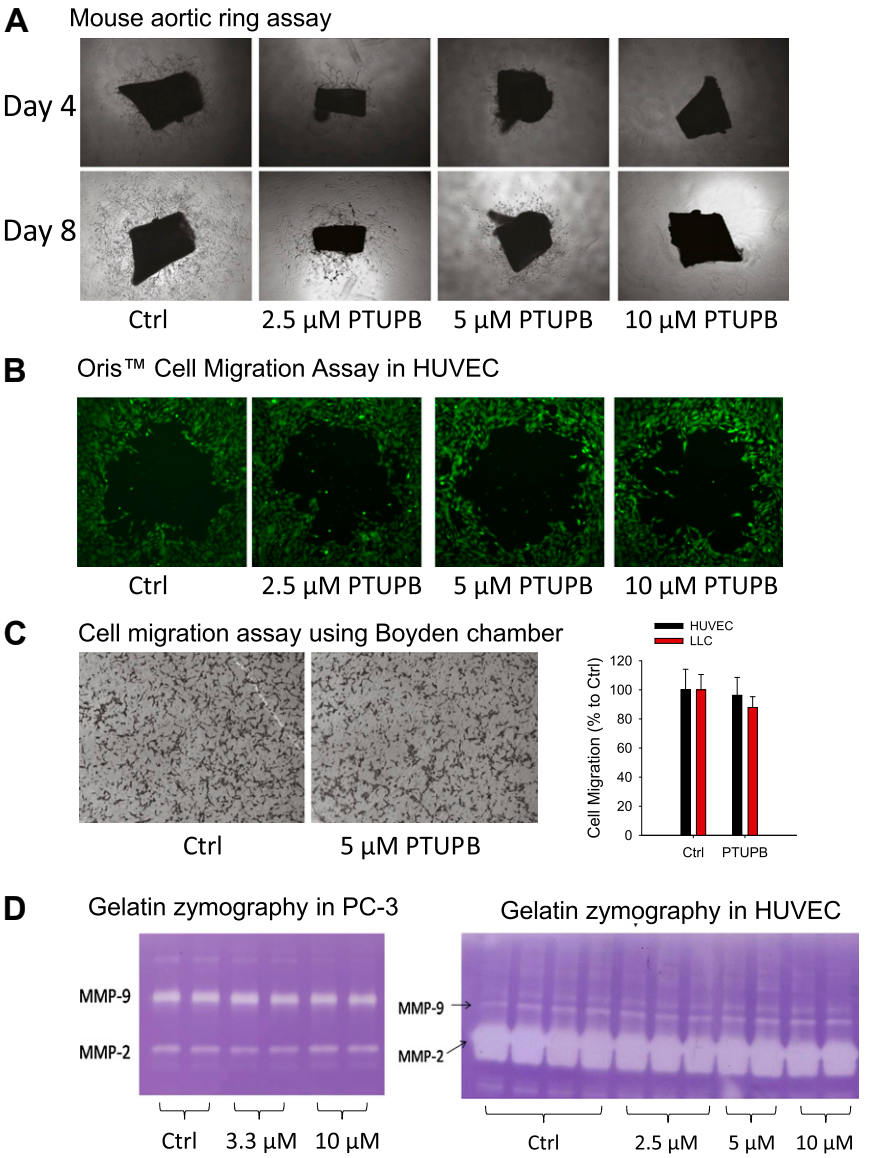
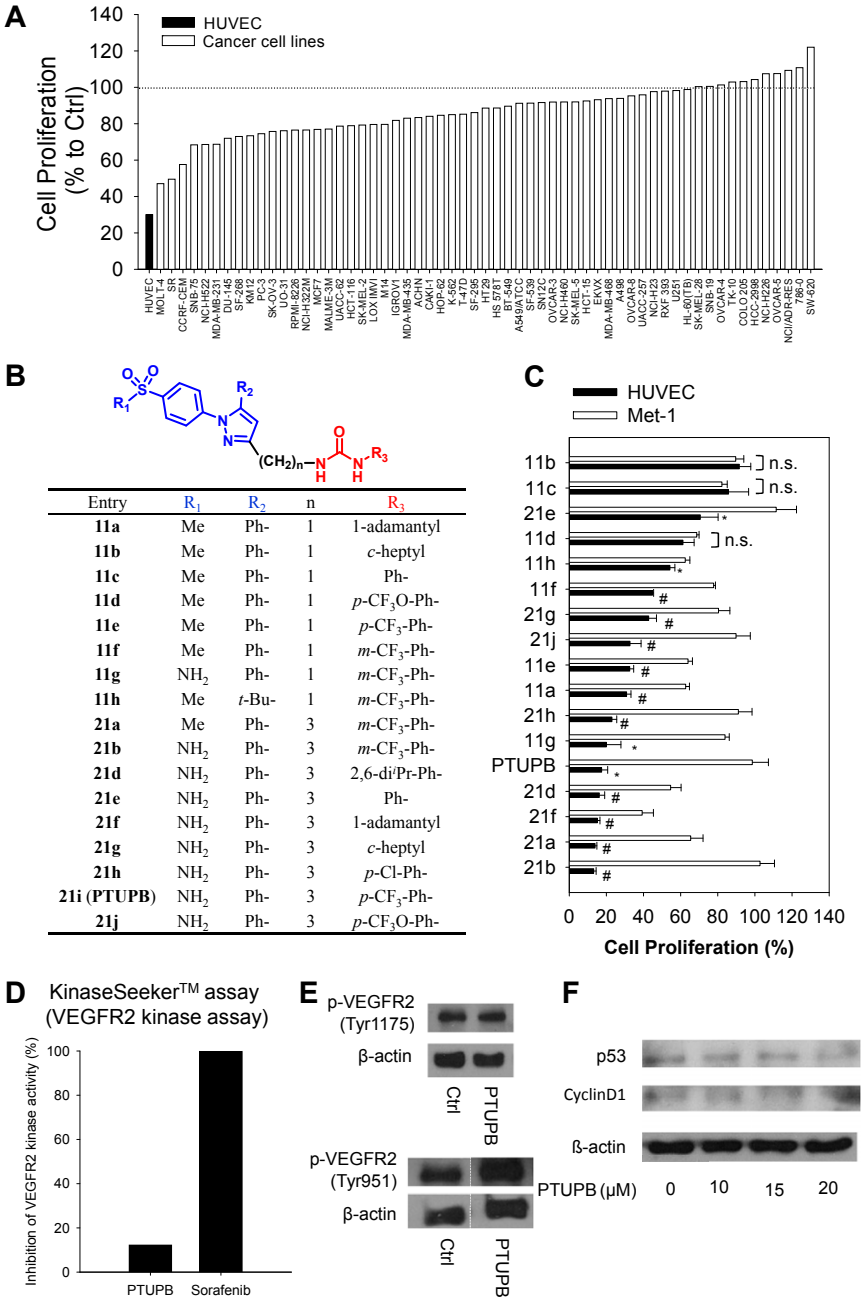


Fig. S3. (*A*) PTUPB inhibited aortic vessel sprouting in a mouse aorta ring assay. (*B*) PTUPB at 2.5–10 μM had no effect on cell migration of HUVECs in an Oris cell migration assay. (*C*) PTUPB at 5 μM had no inhibitory effect on cell migration of HUVECs or LLC cells in a Boyden chamber assay. (*D*) PTUPB had no effect on matrix metalloproteinase (MMP) activities after 24 to 72 h of treatment in HUVECs or human prostate cancer (PC-3) cells.



Entry	R_1	R_2	n	R_3
11a	Me	Ph-	1	1-adamantyl
11b	Me	Ph-	1	*c*-heptyl
11c	Me	Ph-	1	Ph-
11d	Me	Ph-	1	*p*-CF_3O-Ph-
11e	Me	Ph-	1	*p*-CF_3-Ph-
11f	Me	Ph-	1	*m*-CF_3-Ph-
11g	NH_2	Ph-	1	*m*-CF_3-Ph-
11h	Me	*t*-Bu-	1	*m*-CF_3-Ph-
21a	Me	Ph-	3	*m*-CF_3-Ph-
21b	NH_2	Ph-	3	*m*-CF_3-Ph-
21d	NH_2	Ph-	3	2,6-di^{i}Pr-Ph-
21e	NH_2	Ph-	3	Ph-
21f	NH_2	Ph-	3	1-adamantyl
21g	NH_2	Ph-	3	*c*-heptyl
21h	NH_2	Ph-	3	*p*-Cl-Ph-
21i (PTUPB)	NH_2	Ph-	3	*p*-CF_3-Ph-
21j	NH_2	Ph-	3	*p*-CF_3O-Ph-

Fig. S4. (*A*) National Cancer Institute-60 human cancer cell line screening shows that PTUPB at 10 μM had minimal inhibition on the proliferation of cancer cells, although it dramatically inhibited proliferation in HUVECs after 2 d of treatment. (*B*) Chemical structures of the dual inhibitors were synthesized in our library (1). (*C*) Besides PTUPB, many other dual inhibitors also selectively inhibited HUVEC proliferation at a dose of 10 μM. (*D*) In a cell-free KinaseSeeker assay, PTUPB at 10 μM had a minimal effect to inhibit VEGFR2 kinase activity, although the positive control sorafenib at 10 μM inhibited 100% of the kinase activity. This assay was carried out by Luceome Biotechnologies. (*E*) PTUPB at 10 μM had no effect on inhibition of VEGF-induced VEGFR2 phosphorylation (phosphorylation at Tyr1175 or Tyr951 of VEGFR2) in HUVECs. HUVECs were serum-starved for 24 h and then treated with 50 ng/mL VEGF and 10 μM PTUPB for 10 min, followed by Western blot analysis. (*F*) PTUPB had little effect on expression of p53 and Cyclin D1 after 24 h of treatment in HUVECs.

1. Hwang SH, et al. (2011) Synthesis and structure-activity relationship studies of urea-containing pyrazoles as dual inhibitors of cyclooxygenase-2 and soluble epoxide hydrolase. *J Med Chem* 54(8):3037–3050.

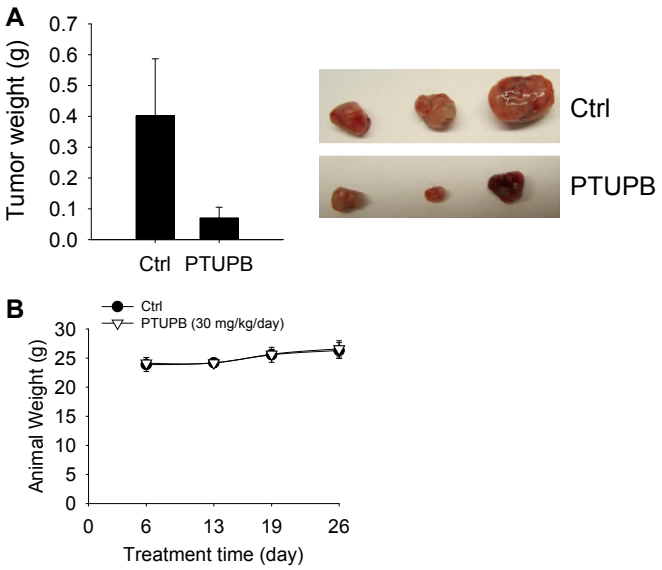


Fig. S5. (*A*) PTUPB (30 mg·kg^{-1}·d^{-1}) inhibited LLC growth after 3 wk of treatment in C57BL/6 mice in a pilot experiment ($n = 3$ mice per group). (*B*) In an NDL tumor experiment, PTUPB has no adverse effect on body weight.

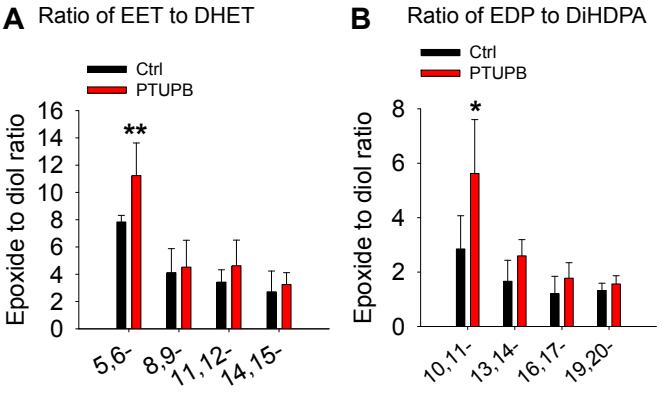


Fig. S6. PTUPB (30 mg·kg^{-1}·d^{-1}) stabilized epoxy fatty acids in plasma in an NDL tumor model in FVB female mice. (*A*) PTUPB increased the ratio of epoxyeicosatrienoic acid (EET; ARA epoxide) to dihydroxyeicosatrienoic acid (DHET; ARA diol) regioisomer in plasma. (*B*) PTUPB increased the ratio of epoxydocosapentaenoic acid [EDP; docosahexaenoic acid (DHA) epoxide] to dihydroxydocosapentaenoic acid (DiHDPA; DHA diol) regioisomer in plasma. The results are expressed as mean ± SD. *$P < 0.05$; **$P < 0.01$.

Table S1. Pharmacokinetic parameters of PTUPB in mice by oral administration (noncompartmental analysis)

Dose, μmol/kg	C_{max}, nM	T_{max}, h	$t_{1/2}$, h	AUC, nM·h
15	140 ± 40	1.5	4.3 ± 1	1,100 ± 200
50	1,950 ± 800	1	3 ± 1.2	6,300 ± 1,200

AUC, area under the curve; C_{max}, peak plasma concentration; T_{max}, time to reach C_{max}.

Table S2. LC-MS/MS analysis of lipid metabolites in the plasma of NDL tumor-bearing mice treated with PTUPB

	PTUPB, nM		Control, nM		
Lipid metabolite	Average	SD	Average	SD	*P* value
9,12,13-TriHOME	15.72	3.49	14.41	2.99	0.5007
9,10,13-TriHOME	12.73	2.83	10.64	2.31	0.1911
PGE_2	0.18	0.03	0.39	0.05	0.0002
15,16-DiHODE	7.18	1.63	5.49	1.46	0.0879
9,10-DiHODE	0.51	0.33	0.49	0.32	0.9335
12,13-DiHODE	1.46	0.73	1.45	0.87	0.9838
17,18-DiHETE	0.43	0.30	0.36	0.07	0.5972
14,15-DiHETE	0.08	0.08	0.05	0.02	0.3745
12,13-DiHOME	33.51	16.15	33.58	14.72	0.9942
9,10-DiHOME	5.03	2.28	5.04	1.93	0.9947
19,20-DiHDPE	3.24	1.80	2.52	0.46	0.3652
14,15-DiHETrE	1.34	1.39	0.97	0.36	0.5521
16,17-DiHDPE	0.59	0.61	0.42	0.11	0.5001
11,12-DiHETrE	0.90	0.94	0.62	0.20	0.4931
13,14-DiHDPE	0.29	0.25	0.22	0.07	0.5592
10,11-DiHDPE	0.21	0.18	0.18	0.08	0.7414
8,9-DiHETrE	0.48	0.16	0.47	0.17	0.9226
EKODE	86.80	81.22	22.74	17.23	0.0881
7,8-DiHDPE	0.48	0.10	0.53	0.09	0.4093
5,6-DiHETrE	0.69	0.17	0.83	0.14	0.1520
4,5-DiHDPE	0.91	0.28	0.69	0.38	0.2781
13-HODE	94.59	79.33	55.83	23.59	0.2780
9-HODE	33.55	23.87	19.56	5.35	0.1914
15(16)-EpODE	32.90	22.45	26.33	11.14	0.5356
15-HETE	9.59	8.14	4.46	1.19	0.1575
9(10)-EpODE	1.51	1.13	0.75	0.20	0.1403
11-HETE	6.08	7.37	1.65	0.83	0.1740
12(13)-EpODE	1.92	1.51	1.56	0.72	0.6153
13-oxo-ODE	3.18	2.09	2.34	1.01	0.3952
15-oxo-ETE	2.77	1.36	1.84	0.38	0.1364
9-oxo-ODE	62.70	43.16	33.16	7.62	0.1298
8-HETE	3.62	2.32	2.07	0.66	0.1482
12-HETE	422.27	400.01	120.47	77.58	0.0997
9-HETE	0.70	0.17	0.71	0.23	0.9576
15(S)-HETrE	1.32	0.68	0.71	0.18	0.0595
12-oxo-ETE	391.20	299.53	199.13	109.36	0.1709
5-HETE	5.39	2.15	3.99	0.24	0.1433
19(20)-EpDPE	3.41	0.98	3.32	0.91	0.8790
12(13)-EpOME	25.11	17.68	19.07	7.85	0.4619
14(15)-EpETrE	2.33	0.46	2.28	0.94	0.9084
9(10)-EpOME	15.10	10.18	6.34	1.46	0.0636
16(17)-EpDPE	0.55	0.19	0.46	0.19	0.4485
13(14)-EpDPE	0.44	0.12	0.33	0.10	0.1259
5-oxo-ETE	2.86	1.60	1.35	0.55	0.0531
10(11)-EpDPE	0.72	0.12	0.50	0.10	0.0091
11(12)-EpETrE	2.11	0.69	1.99	0.21	0.7030
7(8)-EpDPE	23.78	22.95	22.33	12.02	0.8942
8(9)-EpETrE	2.02	0.95	1.74	0.28	0.4968
5(6)-EpETrE	8.35	3.30	6.48	0.79	0.2076