ONLINE DATA SUPPLEMENT

MATERIALS AND METHODS

sEH inhibitors (sEHIs)

Two sEHIs, 1-adamantan-1-yl-3-(5-(2-(2-ethoxyethoxy)ethoxy)pentyl)urea (**AEPU**) and *trans*-4-[4-(3-adamantan-1-ylureido)cyclohexyloxy]benzoic acid (***t*-AUCB**) were used in our study (see Fig. 1 for the structure of the compounds), and were synthesized as previously described [1, 2]. The synthesis, analytical data and the measurement of water solubility for AEPU has previously been published [1, 2].

Measurement of water solubility of t-AUCB

t-AUCB (3 mg) was suspended in 2 mL of nanopure water in a 4 mL glass vial by strong mixing on a Vortex mixer for 5 min and then followed by sonication for 1 h in a 40 ºC water bath. The suspension was then shaken at 250 r.p.m. with an L.E.D. orbit shaker (Lab-Line Instruments, Inc. Melrose Park, IL) at room temperature for 2 days. The suspension was allowed to sit for 1 day at room temperature, and then centrifuged at 14,000 r.p.m. for 15 min. An aliquot of supernatant was diluted with methanol containing 1-cyclohexyl-ureido-3-dodecanoic acid (CUDA, 100 ng/mL) as an internal standard (1000~5000 fold) for LC-MS/MS analysis. The inhibitor was analyzed in triplicate. The water concentration of sEH inhibitors was determined using a UPLC-MS/MS method which was validated to assure acceptable accuracy and precision (accuracy more than 95% with RSD less than 10%). Specifically, chromatographic separation was performed on an ACQUITY ultra performance liquid chromatography (UPLC) instrument equipped with a 2.1 X 50 mm ACQUITY UPLC BEH C_{18} 1.7 μm column (Waters, Milford, MA) held at 25 ºC. The solvent system consisted of water/acetonitrile/formic acid (899/100/1

v/v, solvent A) and acetonitrile/water/formic acid (899/100/1 v/v; solvent B). The gradient was begun at 30% solvent B and was gradually increased in a linear manner to 100% solvent B in 5 min. This was maintained for 3 min, then returned to 30% solvent B in 2 min. The flow rate was 0.4 mL/min. The injection volume was 5 μL and the samples were kept at 4 ºC in the auto sampler. Analytes were detected by positive mode electrospray ionization tandem quadrupole mass spectrometry in multiple reaction-monitoring mode (MRM) on a Quattro Premier Mass Spectrometer (Waters, Milford, MA). The flow rate of nitrogen gas was fixed for the cone gas flow of 50 L h^{-1} and the desolvation gas flow of 650 L h^{-1}. Electrospray ionization was performed with a capillary voltage set at 1.00 kV and an extractor fixed at 3.0 V. The source temperature was set at 120 ºC and the desolvation temperature at 300 ºC, respectively. Collision gas (argon) was set at 3.0 X 10^{-3} Torr. Cone voltage and collision voltage were optimized by acquisition of precursor and production ions, respectively. The water solubility of t-AUCB was 160 mg/L.

Measurement of the concentrations of sEHIs in the plasma

Measurement of the blood concentrations of AEPU has previously been described [1]. Blood samples were taken during the day. Separate studies indicated that when sEHIs were administered in drinking water that the blood concentrations were approximately two fold higher at night than during the day due to increased nocturnal water consumption. Plasma concentrations of *t*-AUCB were as follows: 55.2±28.3 nM for treated MI animals (n=6), 36.1±22.4 nM for treated sham animals (n=6) and the plasma concentrations were under the quantitative limit for the no treatment group (N=12, $p<0.05$ comparing treatment *vs.* no treatment groups). The water intake of the mice averages ~6-7ml/day/mouse. There were no significant differences in the daily water intake between groups.

Myocardial infarction model in mice

All animal care and procedures were approved by the University of California, Davis Institutional Animal Care and Use Committee. Myocardial infarction (MI) model in mice was created using procedure as previously described [3]. Briefly, 10-week old male C57Bl/6J mice (Charles River, Wilmington, MA) were anesthetized with intraperitoneal ketamine 80 mg/kg and xylazine 6 mg/kg. Intubation was performed perorally and mechanical ventilation was initiated. An oblique 4-mm incision was made 4 mm away from the left sternal border in the 3^{rd}-4^{th} intercostal space. The chest retractor was inserted and the heart which was partially covered by the lung was then visualized. The pericardium was gently picked up and pulled apart. The left anterior decending (LAD) coronary artery was then visualized and ligated 1–2 mm below the tip of the left auricle in its normal position. Occlusion was confirmed by the change of color of the anterior wall of the LV and maintained for 45 minutes after which the occlusion was released. Mice were randomized 3 days before surgery to receive two different sEHIs (Fig. 1**a**), AEPU in drinking water (100 mg/L) or *t*-AUCB (15 mg/L)[2] in the drinking water or water alone. For *t*-AUCB, the compound was soaked in drinking water for 2 hours followed by 5 minutes of sonication. The procedure for AEPU has previously been described.[1] The sham-operated mice underwent the same procedure without tying the suture but moving it behind the LAD artery.

Morphometric and histologic analyses

Hearts were excised and retrogradely perfused with phosphate-buffered solution to wash out blood and fixed in 4% formalin overnight. Hearts were then embedded in paraffin and serial LV sections of ~ 5 μm in thickness were taken perpendicular to the longitudinal axis starting from where the ligation was. Tissue sections were stained with Masson's trichrome. The fraction of infarct area, which is the ratio of infarction size to total LV area, was calculated by

computerized planimetry (NIH Image J) of digital images of three Masson's trichrome-stained serial LV sections taken at 0.5- to 1.0-mm intervals along the longitudinal axis as previously described [4]. Measurements were performed by an observer blinded to the treatment groups.

Capillary density in the infarct border zone (viable myocardium adjacent to the infarcted region) was determined by using immunohistochemitry of the cardiac tissue sections [5]. Paraffin sections of mouse hearts (4 μm in thickness) were treated with xylene and ethanol. After re-hydration, heat-induced antigen retrieval was performed in 10 mM sodium citrate for 20 minutes. The sections were then blocked overnight in 10 % normal goat serum in PBS at 4 ºC, after which the primary antibody (anti-CD31 antibody, Abcam, Cambrige, MA) was used to stain for endothelial cells. Primary antibody in PBS containing 10 % normal goat serum was added for 30 min. The sections were then treated with 3% H_2O_2 in PBS for 30 minutes followed by biotinylated secondary antibody. After washing for 5 min in PBS, they were incubated in VECTASTAIN ABC reagent (Vector Laboratories) for 30 minutes. The sections were then incubated in peroxidase solution. The sections were rinsed, counterstained with hematoxylin and mounted. A size criterion of 10 μm was used to exclude small arterioles and venules. Three tissue sections from each animal were used for the measurement of capillary density. All measurements were done in a blinded fashion. Capillary density was expressed as the mean of capillaries/mm^2.

Analysis of cardiac function by echocardiography

Echocardiograms to assess systolic function were performed using M-mode and two-dimensional measurements. The measurements represented the average of six selected cardiac cycles from at least two separate scans performed in random-blind fashion with papillary muscles used as a point of reference for consistency in level of scan. End diastole was defined as the maximal left ventricle diastolic dimension and end systole was defined as the peak of

posterior wall motion. Fractional shortening (FS), a surrogate of systolic function, was calculated from left ventricle dimensions as follows: FS = ((EDD - ESD)/EDD) x100%, where EDD and ESD represent end-diastolic and end-systolic dimension, respectively.

In vivo electrophysiologic studies in mice

In-vivo electrophysiologic studies *were* performed as previously described [6, 7]. Standard pacing protocols were used to determine the electrophysiologic parameters, including sinus node recovery time, atrial, AV nodal, and ventricular refractory periods and AV nodal conduction properties. Each animal underwent an identical pacing and programmed stimulation protocol.

To induce atrial and ventricular tachycardia and fibrillation, programmed extrastimulation techniques and burst pacing were utilized. Programmed right atrial and right ventricular double and triple extrastimulation techniques were performed at 100-ms drive cycle-length, down to a minimum coupling interval of 10 ms. Right atrial and right ventricular burst pacing were performed as eight 50-ms and four 30-ms cycle length train episodes repeated several times, up to a maximum 1-min time limit of total stimulation. For comparison of the inducibility in each mouse, programmed extrastimulation techniques and stimulation duration of atrial and ventricular burst pacing were the same in all mice. Sustained atrial or ventricular arrhythmias were defined as atrial arrhythmias lasting longer than 30 seconds. Reproducibility was defined as greater than one episode of induced atrial or ventricular tachycardia.

Electrophysiologic studies

Cardiac myocytes were isolated from left ventricular free wall (LVFW) remote from the infarcted area using protocol previously described for MI model in rats [8-11]. Due to the known electrophysiologic heterogeneity in various regions of the heart, we used only LV free wall cells

for our electrophysiologic recordings. Briefly, hearts were removed, and retrogradely perfused with collagenase B (Boehringer Mannheim) and 3 mg protease (type XIV, 4.5units/mg, Sigma). At the end of the collagenase perfusion, the atria were removed, the LVFW was dissected out and areas of infarct were discarded.

APs were recorded at room temperature using the perforated-patch technique [12]. All other experiments were performed using the conventional whole-cell patch-clamp technique [13] at room temperature. For AP recordings, the patch-pipettes were backfilled with amphotericin (200 μg/ml). The pipette solution contained (mM) K-glutamate 120, KCl 25, $MgCl_2$ 1, $CaCl_2$ 1, HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid) 10, pH 7.4 with KOH. The external solution contained NaCl 138, KCl 4, $MgCl_2$ 1, $CaCl_2$ 2, NaH_2PO_4 0.33, glucose 10, HEPES 10, pH 7.4 (NaOH).

For K^+ current recording, the external solution contained (mM) NMG 130, KCl 5, $CaCl_2$ 1, $MgCl_2$ 1, Nimodipine 1 μM, glucose 10, HEPES 10, pH 7.4 with HCl. The pipette solution contained (mM) KCl 140, Mg-ATP 4, $MgCl_2$ 1, EGTA 10, HEPES 10, pH 7.4 with KOH. For whole-cell Ca^{2+} current measurement, the external solution contained (mM) N-methyl glucamine 140, CsCl 5, $CaCl_2$ 2, $MgCl_2$ 0.5 glucose 10, HEPES 10, pH 7.4 with HCl. The pipette solution contained (mM) CsCl 125, TEA 20, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA; Molecular probes, OR) 0.05, Mg-ATP 4, HEPES 10, pH 7.3 with CsOH. All experiments were performed using 3 M KCl agar bridges. Cell capacitance was calculated as the ratio of total charge (the integrated area under the current transient) to the magnitude of the pulse (20 mV). Currents were normalized to cell capacity to obtain the current density. The series resistance was compensated electronically. In all experiments, a series resistance compensation of ≥90% was obtained. Currents were recorded using Axopatch 200B amplifier (Axon

Instrument), filtered at 10 kHz using a 4-pole Bessel filter and digitized at sampling frequency of 50 kHz. Data analysis was carried out using custom-written software and commercially available PC-based spreadsheet and graphics software (MicroCal Origin version 6.0).

Metabolomic Profiling of Oxylipins

Plasma samples stored at -80 ºC were thawed at room temperature. Aliquots of plasma (200 µL) were spiked with a set of odd chain length analogs and deuterated isomers of several target analytes including hydroxyeicosatetraenoic acids (HETEs), prostaglandins, thromboxanes, epoxides (EpOMEs and EETs), and diols (DHOMEs and DHETs) contained in 10 µL of methanol, and then were extracted by solid phase extraction using Oasis HLB cartridges (Waters, Milford, MA). The HLB columns (1cc, 60mg) were washed with 2 mL methanol and pre-conditioned with 2 mL water/methanol/acetic acid (95/5/0.1, v/v). Samples were then mixed with 200 µL of the pre-conditioning solution and loaded onto the column. The loaded column was then washed with 2 mL of the pre-conditioning solution and then dried for 5 min *in vacuo*. Target analytes were then eluted with 2 mL of ethyl acetate. The collected eluents were evaporated to dryness using a centrifugal vacuum concentrator and re-dissolved in 40 µL of methanol. The spiked samples were vortexed for 1 min, centrifuged at 14,000 r.p.m. for 5 min, and then transferred to analytical vials containing 150 µL inserts for analysis.

The oxylipin profiling was performed using a modification of a previously published method [14]. The separation of plasma oxylipins was conducted in a Shimadzu LC-10AD$_{VP}$ instrument (Shimadzu Corp., Kyoto, Japan) equipped with a 2.1 mm X 150 mm Pursuit XRs-C18 5 µm column (Varian Inc, Palo Alto, CA) held at 40 ºC. A gradient of water containing 0.1% acetic acid (v/v, solvent A) and acetonitrile/methanol/acetic acid (800/150/1, v/v; solvent B) was used to elute the column with the flow rate of 0.4 mL min^{-1} (SI Table 1). The injection volume

was 10 μL and the samples were kept at 10 ºC in the auto sampler. Analytes were detected on a 4000 QTRAP (Applied Biosystems, Foster City, CA) hybrid, triple-quadrupole, and linear ion trap mass spectrometer equipped with a Turbo V ion source and operated in negative MRM mode. The source was operated in negative electrospray mode and the QTRAP was set as follows: CUR= 20 psi, GS1=50 psi, GS2=30 psi, IS=-4500 V, CAD= HIGH, TEM=400°C, ihe=ON, DP= - 60 V. The collision energies used for CAD ranged from -18 to -38 eV.

Measurement of Plasma Cytokine Levels

Plasma samples were collected 3 weeks after sham or MI operation and stored at -70°C until assayed. Plasma cytokine levels were analyzed using a Cytometric Bead Array kit (CBA mouse inflammation kit, BD Biosciences). Briefly, thawed plasma samples were mixed for 2 hours at room temperature with florescence-labeled capture beads with the PE detection reagents to measure the concentrations of Interleukin-6 (IL-6), Interleukine-1β (IL-1β), Interleukin-10 (IL-10), Monocyte Chemoattractant Protein-1 (MCP-1), Tumor Necrosis Factor-α (TNF-α) and Interleukin-12p70 (IL-12 p70). Samples were then washed with washing buffer and analyzed on a FACScan flow cytometer (BD Immunocytometry Systems). Data were analyzed using BD Cytometric Bead Array Analysis software (BD Immunocytometry Systems).

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling (TUNEL)

Hearts from sham and treated as well as untreated mice were fixed 24-48 hours in 4% neutral-buffered paraformaldehyde and later subjected to paraffin embedding and serial sectioning (5-μm). *In situ* Cell Death Detection Kit, TMR red (Roche Diagnostics), was used for the detection of apoptotic cells. Cardiac sections were first dewaxed, rehydrated, fixed and treated with proteinase K before being permeabilized in 0.01% Triton-X-100.

Permeabilized sections were then incubated with TUNEL reaction mixture containing

terminal deoxynucleotidyl transferase (TdT), unlabeled dNTPs and TMR labeled dUTPs under optimized conditions and ratio in a humid chamber at 37°C for 1 hour. This was immediately followed by incubation (30 minutes, room temperature) with FITC labeled lectin from *Triticum vulgaris* (Sigma) for membrane staining. The nuclei were counter-stained by DAPI present in the mounting media (Vectashield, Vector Laboratories). Three independent experiments were performed, with each group in triplicate. The apoptotic and non-apoptotic nuclei were visualized using a confocal microscope (Pascal Zeiss Confocal Microscope) and digital images were acquired. At least 1,500 DAPI-stained cells in multiple random fields were counted in each group to assess the fraction of apoptotic cells determined by dividing the number of TUNEL-positive cells (red) by the total number of DAPI-positive cardiac myocyte nuclei (blue).

Reference

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Figure Legends

Fig. S1. *Beneficial effects of sEHI (AEPU) in a mouse MI model.* ***a****, Histologic sections (H/E stain) from MI mice treated with AEPU compared to MI alone or sham-operated hearts. Animals were sacrificed after three weeks of follow up.* ***b,*** *Examples of two-dimensional and M-mode echocardiography in the MI mouse model with sham operation, fMI and MI treated with AEPU after three weeks of treatment showing evidence of cardiac failure with chamber dilatation in MI mice. AEPU prevented the development of chamber dilatation in MI mice.* ***c****, Summary data for fractional shortening (%) in the three groups of animals. Data shown are mean ± s.e.m., n = 8-12 for each group,* $^{*}p<0.05$ *comparing treated vs. untreated MI animals.*

Fig. S2. *Histologic sections (Sirius Red stain for collagen) of sham-operated and MI mouse hearts, showing infarct area with scarring at 3 weeks in the MI mouse. Treatment of MI mice with t-AUCB in drinking water decreased the scarring and collagen content.*

Fig. S3. *Assessment of the expression of hypertrophic markers using Western blot analysis and polyclonal antibodies against brain natriuretic peptide (BNP). Left ventricular tissues remote from the infarct zone were used from MI mice treated for 3 weeks with t-AUCB compared to MI*

alone or sham-operated hearts. Monoclonal mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was used to control for loading.

Fig. S4. *Capillary density of the border zone.* **a,** *Photomicrographs of histologic sections of border zone from sham, MI and MI mice treated with t-AUCB for 3 weeks at low (upper panels) and high magnification (middle panels). Lower panels are negative control with secondary antibody only. B, Summary data for capillary density (capillaries/mm^2) from the three groups of animals.*

Fig. S5. Plasma Levels of Oxylipin. *Selected oxylipin levels using oxylipin profiling from sham, MI and MI treated with AEPU at 3 weeks of follow up (*$p<0.05$ *comparing sham or treated MI groups to MI alone) including epoxyoctadecenoic acids (EpOMEs), epoxyeicosatrienoic acids (EETs), thromboxane B2 (TXB2), prostagladin F2a (PGF2*α*).*

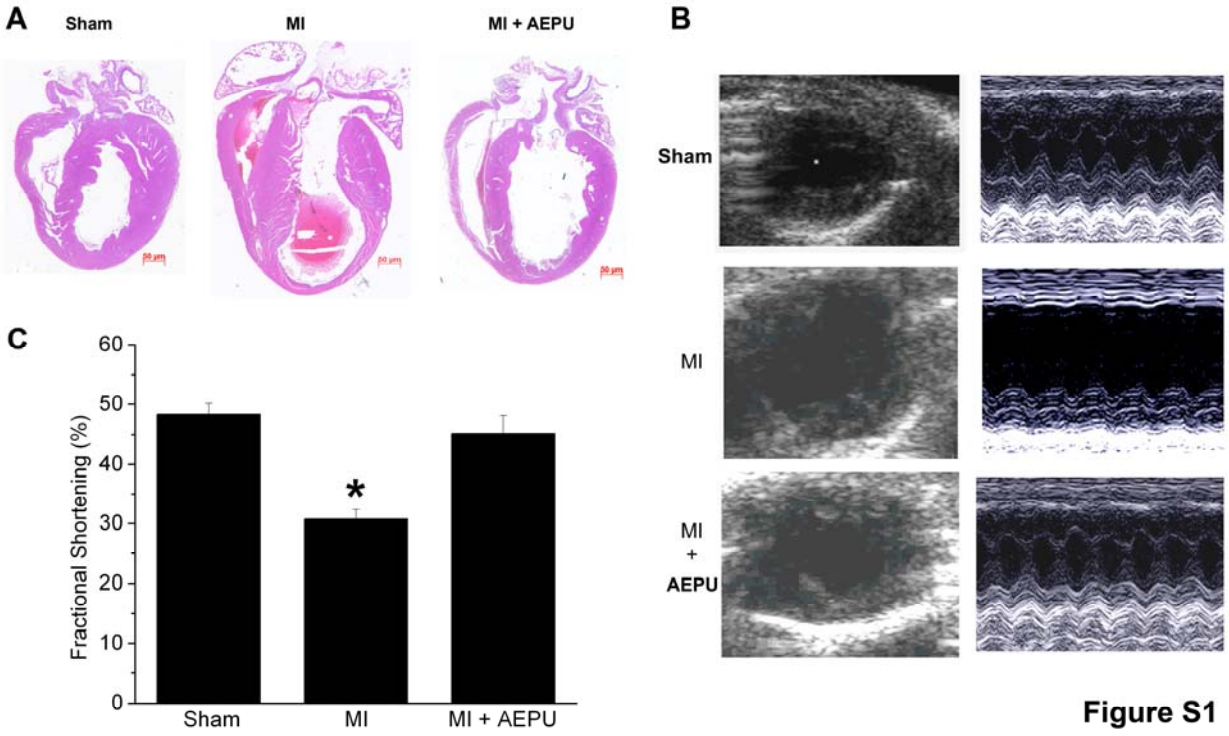

Figure S1

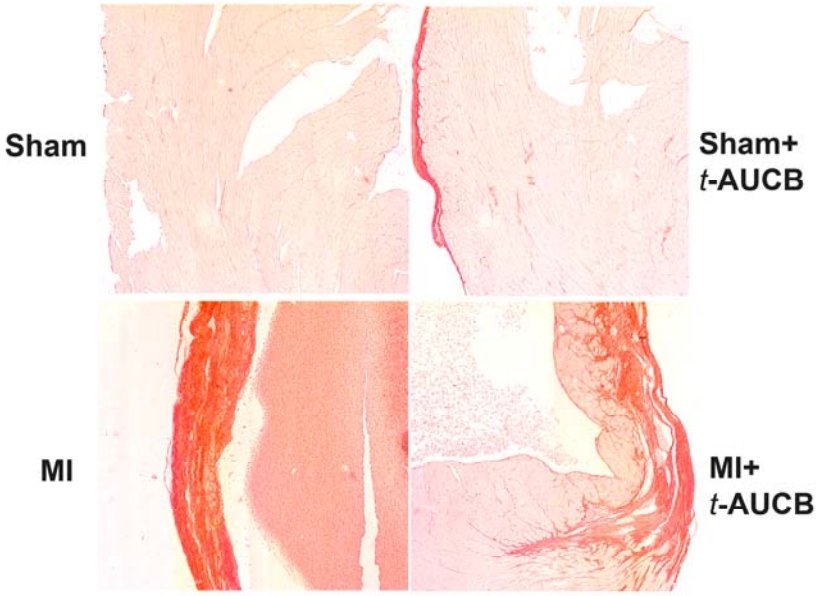

Figure S2

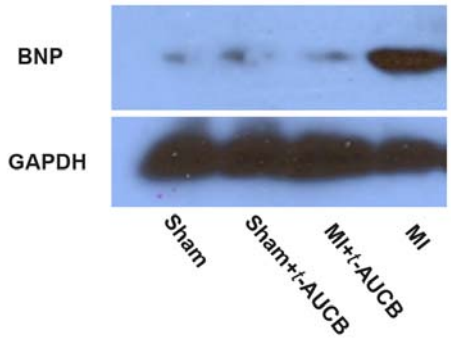

Figure S3

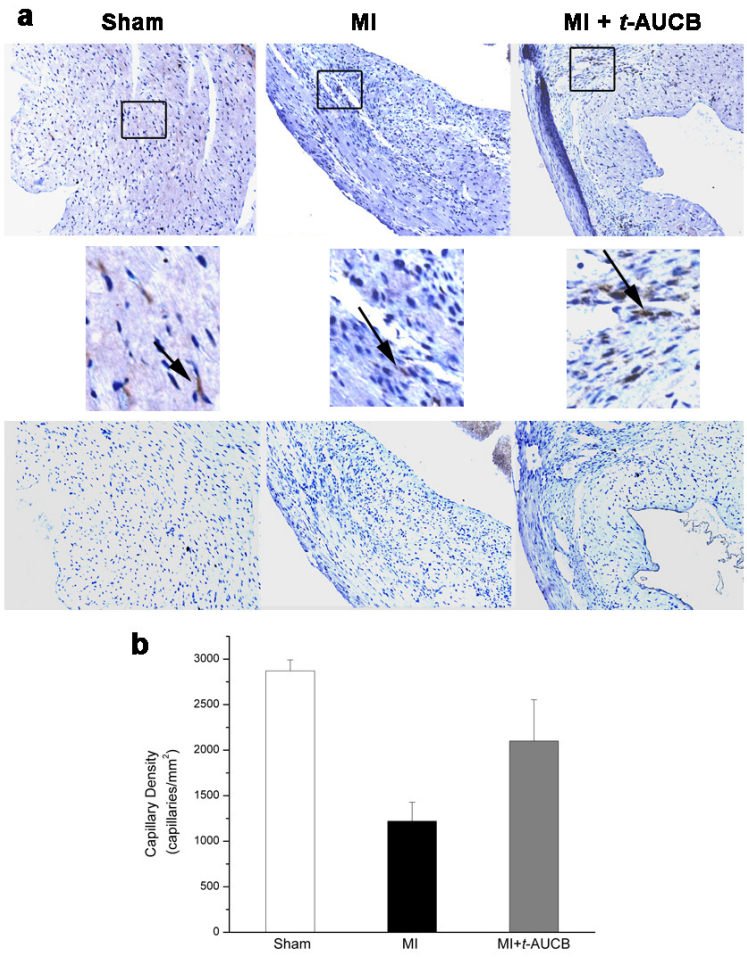

Figure S4

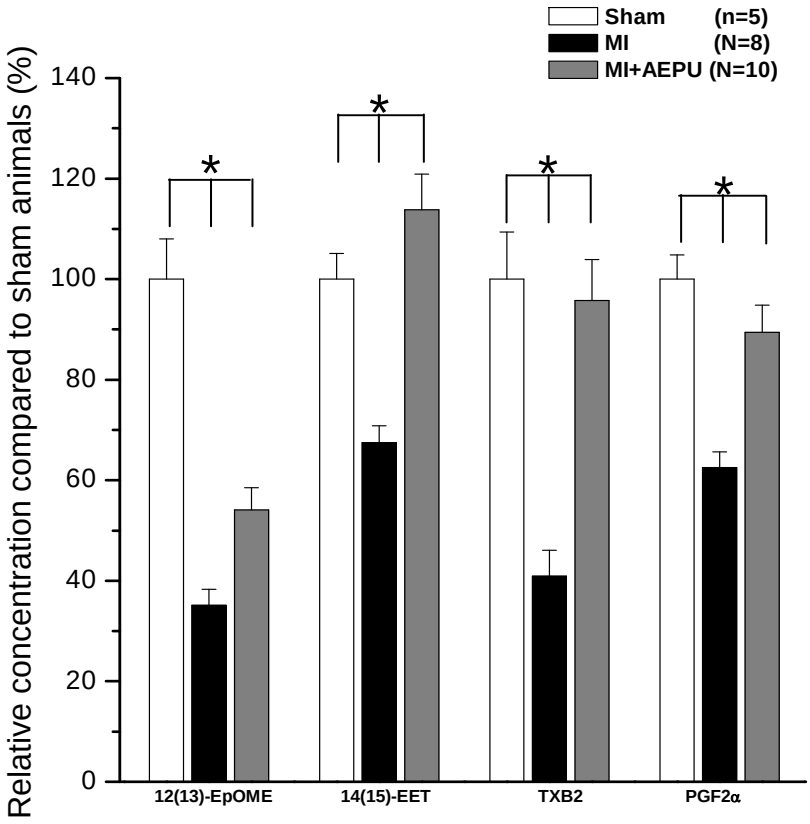
Sham (n=5)
MI (N=8)
MI+AEPU (N=10)
Relative concentration compared to sham animals (%)
140
120
100
80
60
40
20
0
*
*
*
*
12(13)-EpOME
14(15)-EET
TXB2
PGF2α

Figure S5

SI Table 1. *In vivo* electrophysiologic studies in MI mice compared to MI mice treated with *t*-AUCB at 3 weeks.

Treatment	**Sham (n=5)**	**Sham+t-AUCB (n=5)**	**MI (n=9)**	**MI+t-AUCB (n=7)**
SCL	189±11	176±3	198±9	176±12
SNRT120	234±14	219±7	258±13	225±20
CSNRT120	45±7	43±6	60±8	49±8
SNRT100	259±12	223±11	255±16	233±22
CSNRT100	70±10	47±11	57±11	57±12
SNRT80	272±21	217±13	249±13	229±18
CSNRT80	83±18	41±14	51±9	53±9
WCL	90±0	90±0	102±3	94±2
AVNERP120	60±0	60±0	77±3	70±4
AERP120	48±2	50±0	61±3	53±3
VERP120	40±0	38±2	67±2*	46±2
VERP100	36±2	38±2	67±2*	46±2
VERP80	36±2	38±2	67±2*	46±2
AF	0/5	0/5	5/9*	0/7
VF	0/5	0/5	3/9*	0/7

Data shown represent mean ± s.e.m.; SNRT, sinus node recovery time; CSNRT, corrected SNRT; WCL, Wenchebach cycle length; AVNERP, AERP and VERP refer to the effective refractory period for the atrioventricular node, atria and ventricles, respectively; AVNERP, AERP and VERP were performed using basic cycle length of 120, 100 and 80 ms; n refers to the number of the animals in the studies; *$p<0.05$.

SI Table 2. The solvent gradient program for chromatographic separation of oxylipin mediators

Total Time (min)	A* (%)	B* (%)
0	85	15
0.75	85	15
1.5	70	30
3.5	53	47
5	46	54
6	45	55
10.5	40	60
15	30	70
16	20	80
17	0	100
20	0	100
21	85	15
25	85	15

***** Buffer A was 0.1% acetic acid in distilled H_2O, and buffer B was a mixture of acetonitril/methanol/acetic acid (85/10/0.1, v/v). The flowing rate was 0.4 mL/min.

SI Table 3. Plasma levels of oxylipin mediators and the ratios of epoxides to diols in currently investigated murine model

Treatment	Sham (n=5)	Sham+*t*-AUCB (n=5)	MI (n=7)	MI + *t*-AUCB (n=7)
12(13)-DiHOME	3.94±2.36	10.67±3.66	9.23±9.27	19.25±19.14
9(10)-DiHOME	28.78±9.44	64.95±23.76	74.16±79.23	102.85±109.88
14(15)-DHET	0.24±0.04	0.38±0.12	0.48±0.29	0.54±0.32
11(12)-DHET	0.17±0.02	0.22±0.10	0.36±0.24	0.35±0.25
8(9)-DHET	0.49±0.11	0.48±0.18	0.49±0.18	0.52±0.16
5(6)-DHET	0.32±0.06	0.22±0.06	0.30±0.09	0.31±0.07
12(13)-EpOME	7.23±3.00	31.93±21.86*,†	5.38±2.56	28.75±11.77*,†
9(10)-EpOME	2.08±0.54	5.84±3.23	2.52±1.96	6.66±5.44
14(15)-EET	0.38±0.05	1.27±0.69	0.34±0.09	1.07±0.19*,†
11(12)-EET	0.31±0.03	0.54±0.22	0.26±0.07	0.50±0.15*,†
8(9)-EET	0.22±0.03	0.55±0.24	0.24±0.08	0.54±0.14*,†
SUM(EpOMEs)	9.31±3.44	37.77±24.97	7.90±4.38	34.51±16.64*,†
SUM(DHOMEs)	32.72±11.74	75.62±27.41	83.89±88.45	122.10±128.91
SUM(EETs)‡	0.91±0.07	2.36±1.13	0.84±0.23	2.11±0.45*,†
SUM(DHETs)‡	0.90±0.09	1.08±0.40	1.33±0.56	1.40±0.69
9(10)-EpOME/9(10)-DHOME	0.07±0.01	0.10±0.05	0.04±0.01*	0.09±0.04†
12(13)-EpOME/12(13)-DHOME	2.08±0.73	3.28±2.28	0.89±0.57	2.83±1.96
14(15)-EET/14(15)-DHET	1.57±0.10	3.24±0.98†	0.90±0.45*	2.55±1.14†
11(12)-EET/11(12)-DHET	1.87±0.27	2.50±0.59†	0.98±0.50*	1.79±0.62†
8(9)-EET/8(9)-DHET	0.46±0.10	1.14±0.14*,†	0.50±0.08	1.07±0.19*,†
SUM(EpOMEs)/SUM(DHOMEs)	0.29±0.06	0.54±0.35	0.14±0.07*	0.54±0.36
SUM(EETs)/SUM(DHETs)	1.01±0.10	2.14±0.42*,†	0.69±0.20*	1.69±0.46*,†

Data present average ± SD from the total individual mouse in each group. * Significant difference from group 1;†, significant difference from group 3. Significant difference ($p<0.05$) was determined by one way ANOVA followed by Tukey or Games-Howell tests for post hoc comparison. ‡, The 5(6)-EET is excluded because of lactone formation during sample preparation, and 5(6)-DHET is also excluded because of the absence of 5(6)-EET in total EETs.