Supporting Information

Indole Alkaloid Derivative B (IADB), a Novel Bi-functional Agent that Mitigates 5-Fluorouracil-Induced Cardiotoxicity

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General. All chemicals were purchased from Sigma Aldrich. Unless otherwise stated, all reactions were run under a nitrogen atmosphere (1 bar). The purity (>97%) of the intermediates and the final products was confirmed using both TLC (Merck silica gel plates of type 60 F254, 0.25 mm layer thickness) and HPLC (Waters, C_{18} column 4.6mm x150 mm). NMR spectra were recorded on a Bruker Advance 500 spectrometer. The EI-MS was determined by Trace MS (American Thermo Finnigan). The ESR spectra were obtained from 10^{-5} mol/L phosphate-buffered saline, using a BRUKER 300-E spectrometer. The conditions of measurements were as follows: center field: 3440 G, sweep width: 100 G, sweep time: 60 s, modulation amplitude: 1.1 G, time constant: 8.2×10^{-2} s, modulation frequency: 100 kHz, microwave frequency: 9.68 GHz, and microwave power: 20 MW.

3*S*-1,2,3,4-Tetrahydro-β-carboline-3-carboxylic acid (**1**): L-Tryptophan (5.0 g; 24.5 mmol) was supplemented with 25 mL of H_2SO_4 (1 mol/L), 80 mL of water, and 8 mL of formaldehyde (36–38%) added. The reaction mixture was stirred at room temperature for 2 h and adjusted to pH 6–7 with concentrated ammonia. The mixture was maintained at 0 °C for 12 h and the precipitate (98.5%) was collected by filtration. After re-crystallization, 3.97 g (75%) of product was obtained as a colorless powder. Mp: 280–282 °C. EI/MS: 217 $[M + H]^+$; ^{1}H NMR (DMSO-d_6): δ/ppm = 10.92 (s, 1H), 9.87 (s, 1H), 7.32 (t, J = 7.5 Hz, 1H), 7.24 (t, J = 7.9 Hz, 1H), 7.02 (d, J = 7.9 Hz, 1H), 6.83 (d, J = 7.7 Hz, 1H), 4.05 (t, J = 4.7 Hz, 1H), 3.77 (dd, J = 10.3 Hz, J = 5.2 Hz, 1H), 3.66 (dd, J = 10.6 Hz, J = 2.5 Hz, 1H), 2.93 (d, J = 10.6 Hz, 2H), 2.87 (s, 1H). Anal. Calcd for $C_{12}H_{12}N_2O_2$: C, 66.65; H, 5.59; N, 12.96. Found: C, 66.45; H, 5.72; N, 12.81.

***N*-Boc-3*S*-1,2,3,4-Tetrahydro-β-carboline-3-carboxylic acid** (**2**): A suspension of 1.1 g (5.0 mmol) of **1** in 15 mL DMF and 1.4 mL of triethylamine was vigorously stirred at room temperature, and supplemented with 1.1 g (7.7 mmol) of Boc–N_3 over a 30min period. The reaction

mixture was stirred at room temperature for 24 h and then at 40 °C for 80 h. Citrate solution (5 mL; 20%) was then added and the solution extracted with ethyl acetate (3 × 30 mL). Combined ethyl acetate fractions were dried over anhydrous $MgSO_4$. The filtrate was dried by evaporation after removal of $MgSO_4$. The residue was crystallized in $CHCl_3$ to yield 1.20 g (76%) of product. Mp: 165–170 °C. TOF/MS: 317 $[M + H]^+$, 339 $[M + Na]^+$, 355 [M + K]; ^{1}H NMR (DMSO-d_6): δ/ppm = 10.89 (s, 1H), 9.87 (s, 1H), 7.35 (t, J = 7.6 Hz, 1H), 7.22 (t, J = 7.9 Hz, 1H), 7.05 (d, J = 7.8 Hz, 1H), 6.86 (t, J = 7.5 Hz, 1H), 4.86 (t, J = 5.1 Hz, 1H), 4.23 (dd, J = 10.3 Hz, J = 4.9 Hz, 1H), 3.99 (dd, J = 10.1 Hz, J = 3.4 Hz, 1H), 2.95 (d, J = 10.3 Hz, 2H), 1.47 (s, 9H). Anal. Calcd for $C_{17}H_{20}N_2O_4$: C, 64.54; H, 6.37; N, 8.86. Found: C, 64.41; H, 6.25; N, 8.74.

***tert*-Butyl (*S*)-3-(((*S*)-1-(benzyloxy)-4-methyl-1-oxopentan-2-yl)carbamoyl)-1,3,4,9-tetra-hydro-2*H*-pyrido-[3,4-*b*]-indole-2-carboxylate (3)**: HOBt (6.0 mmol) was added to 4.6 mmol of **2** in 30 mL of anhydrous THF at 0 °C. After stirring for 10 min, 6.0 mmol of EDC and a catalytic amount of HOBT was added. A suspension of 5.0 mmol HCl·Leucine benzyl ester in 3 mL anhydrous THF was adjusted with *N*-methyl morpholine to pH 8–9 and then stirred at room temperature for another 20 min. This mixture was then added to solution of **2**, and the reaction mixture stirred at 0 °C for 2 h and then continued stirring at room temperature for 16 h. Following evaporation, the residue was dissolved in 30 mL of ethyl acetate. The solution was washed extensively with 5% sodium bicarbonate, 5% citric acid, and saturated sodium chloride, and the organic phase separated and dried over anhydrous sodium sulfate. Following filtration and evaporation under vacuum yielded the title compound. ESI-MS (m/e) 520 $[M+H]^+$; ^{1}H NMR (DMSO-d_6) δ = 10.85 (d, J = 18.9Hz, 1H), 8.42 (m, 1H), 7.33 (s, 5H), 7.25 (m, 2H), 7.05 (m, 2H), 5.13 (m, 1H), 4.95 (m, 2H), 4.72 (d, J = 18.9Hz, 1H), 4.56 (m, 1H), 3.26 (m, 1H), 3.07 (m, 1H), 1.57 (9m, 3H), 1.45 (s, 9H), 0.85 (s, 3H), 0.77 (s, 3H). ^{13}C-NMR (DMSO-d_6): δ = 172.5, 172.0,

155.5, 136.5, 136.3, 131.1, 128.8, 128.4, 128.2, 126.8, 121.1, 118.8, 117.8, 111.3, 104.4, 103.8, 80.1, 80.0, 66.4, 53.5, 52.2, 50.8, 50.4, 33.8, 28.4, 24.9, 24.6, 24.2, 23.4, 23.2, 15.7.

Benzyl (*S*)-2-((*S*)-3,3-dimethyl-1-oxo-5,6,11,11a-tetrahydro-1*H*-imidazo [1',5':1,6] pyrido [3,4-*b*]indol-2(3*H*)-yl)-4-methyl-pentanoate (**4**): To a solution of compound **3** (2.0 mmol) in EtOAc (10 mL) at 0 °C, HCl (4N, 10 mL) was added. The reaction mixture was stirred at 0 °C for 2h followed by evaporation. The residue was triturated with ether (20mL) to give the colorless powder, which was then dissolved in a mixture of ethanol (40mL) and acetone (10 mL). The pH was adjusted to 9 with N-methyl morpholine. The reaction mixture was then continued stirring at r.t. until TLC analysis indicated complete disappearance of compound **3**. Following evaporation, the residue was dissolved in 50 mL ethyl acetate. The solution was washed with saturated sodium chloride (30mL x3). The organic phase was separated and dried over anhydrous sodium sulfate. The crude residue was further purified by flash column chromatography (petroleum ether/acetone, 4/1) to give the title compound (Yield: 41.5%). ESI-MS (m/e): 460 $[M+H]^+$; Mp: 190-192 °C; ^{1}H NMR ($CDCl_3$): δ= 7.90 (s, 1H), 7.55 (d, *J*= 8.2Hz, 1H), 7.35 (m, 6H), 7.16 (m, 2H), 5.22 (m, 2H), 3.98 (m, 2H), 3.86 (d, *J* =13.6 Hz, 1H), 3.49 (m, 1H), 3.22 (m, 1H), 2.79 (m, 2H), 1.62 (m, 1H), 1.47 (s, 3H), 1.26 (s, 3H), 0.97 (m, 6H). ^{13}C NMR ($CDCl_3$) δ = 171.8, 170.6, 136.3, 135.3, 131.0, 128.7, 128.7, 128.5, 128.3, 127.3, 121.7, 119.7, 118.2, 110.7, 108.5, 78.9, 66.8, 61.1, 57.4, 41.6, 34.1, 29.7, 25.6, 25.0, 23.9, 18.9, 16.6, 11.2.

(*S*)-2-((*S*)-3,3-dimethyl-1-oxo-5,6,11,11a-tetrahydro-1*H*-imidazo[1',5':1,6]pyrido[3,4-*b*]indol-2(3*H*)-yl)-4-methylpentanoic acid (**5**): To a solution of compound **4** (200mg) in methanol, Pd/C (50mg) was added and the solution stirred under hydrogen atmosphere at room temperature. The reaction mixture was filtered and evaporated *in vacuo*. The residue was further purified by flash column chromatography to give the title compound as a light-yellow powder ($CHCl_3$/

MeOH/HCOOH, 20/1/0.10). ESI-MS (m/e): 368 $[M-H]^-$; Mp: 236-237°C; $[\alpha]_D^{20}$= -72.22° (c =0.60, CH_3OH); ^1H NMR (DMSO-d_6) δ= 11.0 (s, 1H), 7.37 (dd, J_1 = 30.5Hz, J_2= 7.7Hz, 2H), 7.05 (m, 2H), 3.95 (m, 2H), 3.82 (d, J = 14.1Hz, 1H), 3.45 (m, 1H), 2.88 (m, 1H), 2.05 (m, 2H), 1.67 (m, 2H), 1.45 (s, 3H), 1.31 (s, 3H), 0.96 (t, 6H); ^{13}C NMR (DMSO-d_6) δ= 172.7, 171.0, 136.5, 133.2, 127.2, 120.9, 118.8, 117.9, 111.4, 106.3, 78.5, 57.2, 53.2, 47.0, 41.8, 38.9, 33.80, 25.81, 25.2, 24.9, 23.8, 23.2, 22.8, 19.7.

Benzyl N^2-((*S*)-2-((*S*)-3,3-dimethyl-1-oxo-5,6,11,11a-tetrahydro-1*H*-imidazo [1',5':1,6] pyrido [3,4-*b*] indol-2 (3*H*)-yl)-4-methylpentanoyl)-N^5-ethylglutaminate (<u>6</u>): To a solution of <u>5</u> (1.5 mmol) in 30 mL of anhydrous THF at 0 °C, L-theanine benzyl ester (1.6 mmol), HOBT (1.5mmol) and EDC (1.6 mmol) were added, respectively. The pH was adjusted with *N*-methyl morpholine to 8, and then stirred at room temperature until TLC analysis indicated the complete disappearance of compound <u>5</u>. Following evaporation, the residue was dissolved in 30 mL of ethyl acetate. The solution was washed with 5% sodium bicarbonate, 5% citric acid, and saturated sodium chloride, and the organic phase separated and dried over anhydrous sodium sulfate. Following filtration and evaporation under vacuum, the crude residue was further purified by flash column chromatography to give the title compound. ESI-MS (m/e): 616 $[M+H]^+$; Mp:101-102°C; $[\alpha]_D^{20}$= -25.23° (c= 0.06, CH_3OH). ^1H NMR (DMSO-d_6) δ= 10.90 (s, 1H), 8.05 (d, J =7.7Hz, 1H), 7.85 (s, 1H), 7.46 (d, J = 7.6Hz, 1H), 7.35 (s, 6H), 7.11-6.99 (m, 2H), 5.15 (s, 2H), 4.42 (s, 1H), 4.01-3.96 (m, 2H), 3.75 (d, J = 7.5Hz, 1H), 3.55 (dd, J = 3.7Hz, J = 9.8Hz, 1H), 3.25 (s, 1H), 3.12-3.02 (m, 2H), 2.93 (s, 1H), 2.97 (s, 1H), 2.21-2.06 (m, 4H), 1.98-1.85 (m, 2H), 1.58 (s, 1H), 1.40 (s, 3H), 1.29 (s, 3H), 1.05-0.88 (m, 9H). ^{13}C NMR (DMSO-d_6) δ= 172.3, 171.8, 171.5, 171.1, 136.7, 136.3, 133.2, 128.9, 128.8, 128.5, 128.3, 127.2, 121.0, 118.9, 117.9, 111.4, 106.5, 79.3,

66.5, 57.5, 56.1, 52.34, 49.1, 41.8, 40.9, 40.6, 40.3, 40.05, 39.8, 39.5, 39.2, 39.1, 33.8, 31.7, 27.2, 25.3, 25.0, 24.1, 23.1, 22.7, 18.5, 15.1.

IADB: To a solution of compound **6** (2.0mmol) in methanol, Pd/C (50mg) was added and the solution stirred under hydrogen atmosphere at room temperature. The reaction mixture was filtered and evaporated *in vacuo*. The crude residue compound **7** was directly subjected to the coupling reaction without further purification. At 0°C, to a solution of compound **7** (1.5mmol) in CH_2Cl_2, EDC (1.6mmol), 4-amino Tempo (1.6mmol), a catalytic amount of HOBT were added. The pH was adjusted to 8-9. The reaction mixture was stirred under argon until TLC analysis indicated the complete disappearance of compound **7**. The solvent was evaporated under vacuo and the crude residue was further purified by flash chromatography to give the target compound, IADB, as an orange powder. ^{1}H NMR (DMSO-d_6) δ= 11.02 (brs), 8.20 (brs), 8.03 (brs), 7.82 (brs), 7.40 (brs), 7.30 (brs), 7.21 (brs), 7.10-6.98 (brs), 4.45 (brs), 3.98 (brs), 3.65 (brs), 2.05-2.10 (brs), 2.90-3.15 (brs), 1.70-1.75 (brs), 1.50 (brs), 0.92-1.15 (brs). The observed paramagnetic broadening was due to the effect of the free radical from the nitroxide moiety. ^{13}C NMR (DMSO-d_6, 125MHz) δ= 174.3, 172.5, 171.5, 170.8, 136.6, 132.5, 128.3, 121.5, 120.1, 119.3, 111.7, 106.2, 79.2, 76.5, 57.5, 53.7, 55.5, 47.6, 41.7, 38.2, 37.8, 32.7, 34.6, 22.8, 28.3, 27.2, 25.6, 24.8, 23.2, 23.0, 22.6, 15.1. HR/MS [M+H]+ calcd, 679.4343, found, 679.4418.

Biochemical assay

Cell culture: All cell lines were obtained from American Type Cell Culture collection (ATCC). HeLa cells were grown in Eagle's Minimal Essential Medium (EMEM) and 10% FBS (Sigma Aldrich, heat inactivated). Human colonic adenocarcinoma HT-29, lung cancer A549, breast cancer cell lines MDA-MB231 were routinely maintained in Dulbecco's modified Eagle medium

(DMEM; Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco). H9C2 cardiac myoblasts were obtained from ATCC and maintained in DMEM medium supplemented with 10% FBS and 1% penicillin-strsptomycin. All cells were grown at 37°C in a humidified 5% CO_2 atmosphere.

Drug treatment in HT-29 cells: HT-29 cells were grown until they reached ~50% confluence, and then the cells were subjected to drug treatment. To assess the effects of the co-treatment with 5-FU and **IADB**, the cells were pre-treated with **IADB** (10 μM) for 12h, and after washing with PBS, cells were treated with 5-FU (5 μM) for another 48h.

Cell proliferation assay: The drug treated HT-29 cells and untreated control cells were cultured in 96-well plates at a density of 5×10^3 cells per cell in 100 μl complete medium. The CellTiter96 ® cell proliferation assay kit was used following the manufacturer's protocol, and cell survival was evaluated using trypan blue exclusion test.[2] All experiments were performed in triplicate wells, and the proliferation of HT-29 cells was calculated as the ratio of each experimental condition to the untreated control cells.

Expression of Green Fluorescent Protein LC3: The green fluorescent protein (GFP) human LC3 fusion protein expressing plasmid pEGFP LC3 was purchased from Life Technologies. Cells (5×10^4 per well) were seeded in six well plates the day before transfection and the plasmid was transfected with FuGENER HD Transfection Reagent. GFP fusion proteins were observed under a laser scanning microscope system. The percentage of GFP LC3 positive cells with GFP LC3 punctate patterning was determined from three independent experiments and the means derived.

Live cell imaging: Cells were grown in 35 mm glass bottom dishes for 24 h in complete media. The media was removed and cells were washed three times with 1× DPBS without Ca^{2+} or Mg^{2+} (Hyclone, Fisher Sci.). Fluorescent probes were incubated with cells in non-FBS media. After each

step, cells were washed with DPBS buffer. Cells were imaged using an Olympus confocal laser scanning microscope.

***In vivo* studies**: All animal tests were performed in compliance with the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health. The assessments described herein were performed based on a protocol reviewed and approved by the Ethics Committee of HeBei Medical University.

Animal Test: All animal tests were performed in compliance with the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health. Male ICR mice (20 ± 2 g) were housed in a 12/12 light/dark cycle at 21 ± 2 °C for 24 h before use. Mice were randomly divided into four groups: (i) *Sham-control group*: intraperitoneally injected with saline for 14 consecutive days; (ii) *5-FU alone group*: mice were intraperitoneally injected with 5-FU (10 mg/kg) once over three consecutive days; (iii) ***IADB** alone group*: mice were intraperitoneally injected with **IADB** (30 mg/kg) for 14 consecutive days; (iv) ***IADB** +5-FU group*: mice were intraperitoneally injected with **IADB** (30mg/kg) for 11 consecutive days, and then intraperitoneally administered 5-FU (10mg/kg) + **IADB** (30mg/kg) once over 3 consecutive days. Upon conclusion of experiments, mice were euthanized via sodium pentobarbital overdose. Heart tissue samples were immediately separated, and blood was rapidly obtained from the ascending aorta. Tissue samples were divided into two sections, one for determination of lipid peroxidation, and the other used for histological examination (Leica CM 1850 UV clinical cryostat) at -30°C.

Histological Analysis: Tissue were mounted in gum tragacanth in the appropriate orientation, and snap frozen in isopentane chilled liquid nitrogen. The frozen tissue sections (4-5 μm) were cut as previously described and stained with haematoxylin-eosin, modified trichrome or NADH followed by light microscopic examination.

Statistical analysis: A two-way ANOVA followed by Scheffé's test was employed using the Origin Program. If differences were observed, the values were then analyzed using Student's *t*-test for paired data. All values were expressed as mean ± SE, and significance was set at the 95th centile.

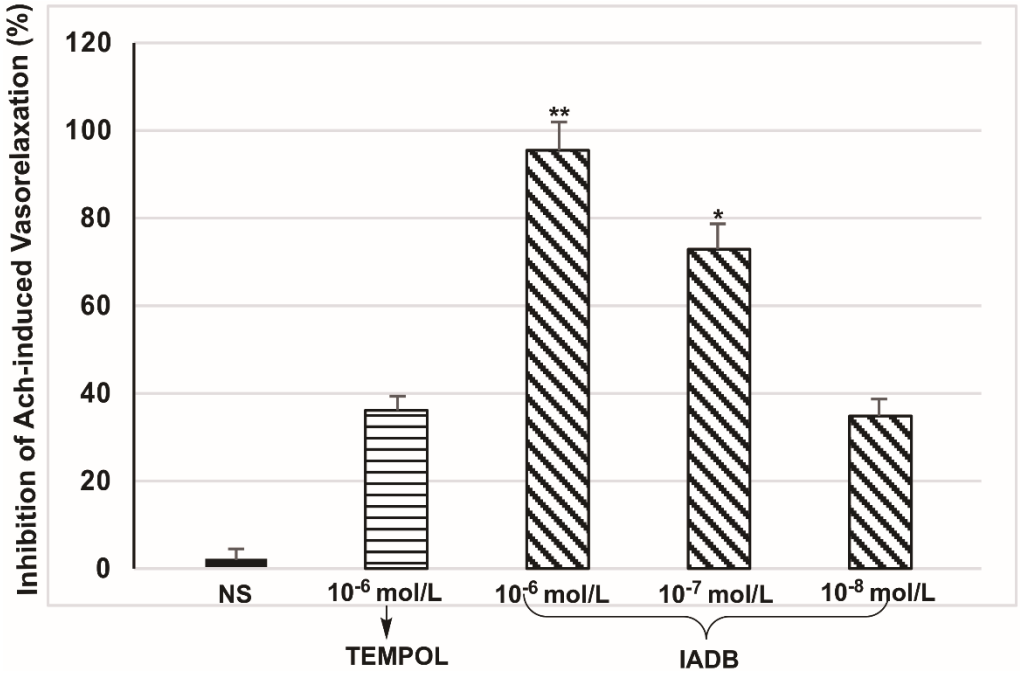

Figure S1. Percent inhibition of Ach-induced vaso-relaxation by TEMPOL and **IADB** (% X±SD). N=6. *: in comparison with TEMPOL, p<0.01; **: in comparison with TEMPOL, p<0.001.

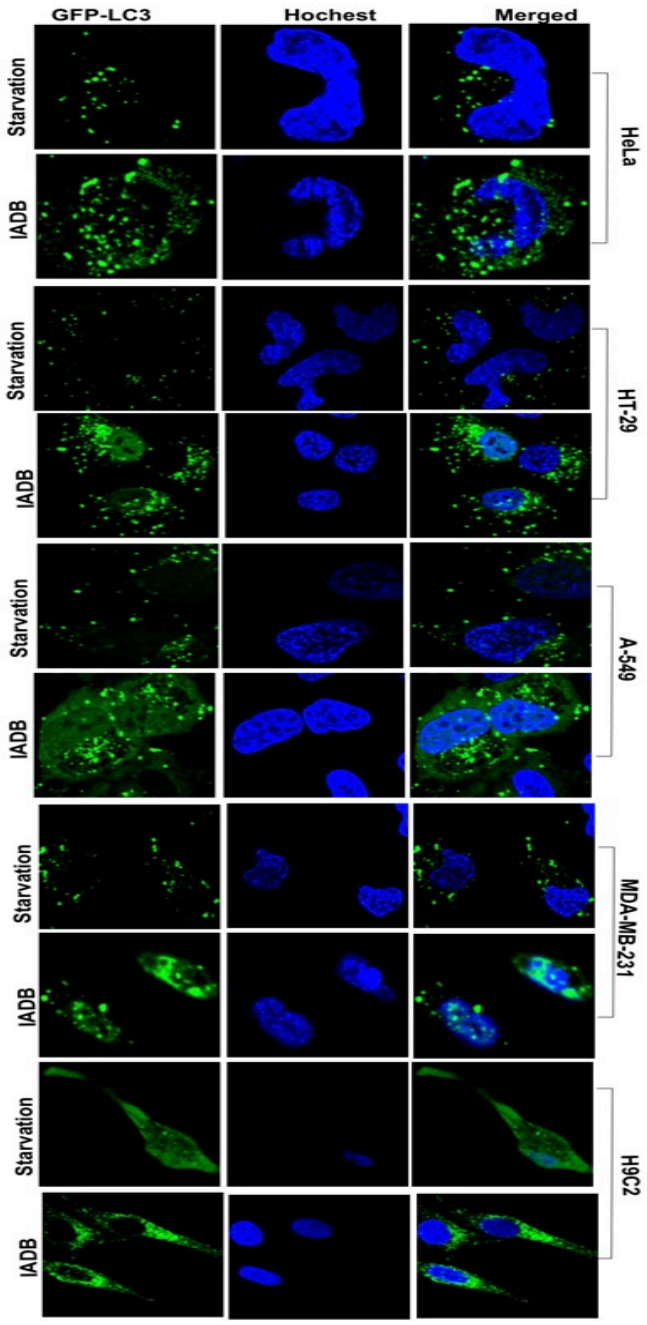

Figure S2. **IADB** induces GFP-LC3 puncta formation in cancer (HeLa, HT-29, A-549, MDA-MB-231) cells and rat normal H9C2 cardiac cells. Cells were transiently transfected with the GFP-LC3 plasmid for 24h and then treated with **IADB** (5 μM) for 24h. Confocal fluorescence images were captured at 60× magnification.

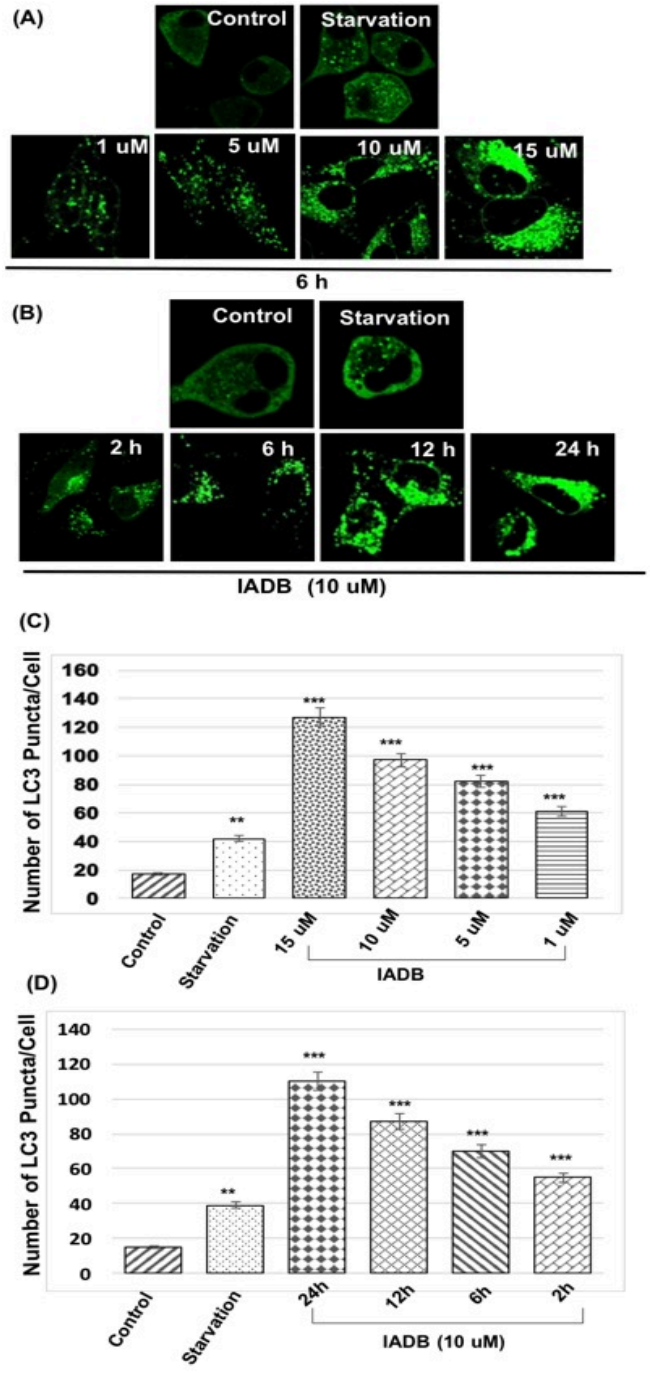

Figure S3. **IADB** induces autophagic vesicle accumulation and the appearance of punctate GFP-LC3 staining in a time- and dose-dependent manner. (**A**) GFP-LC3 stably transfected HeLa cells were treated with increasing amounts (1-15 μM) of **IADB** for 6 h; (**B**) HeLa/GEP-LC3 cells were

treated with 10 μM **IADB** for the indicated time periods (2-24h); (**C**)&(**D**) Statistical analysis of the number of GFP-LC3 puncta structures per cell in each treatment scheme from (**A**)&(**B**).

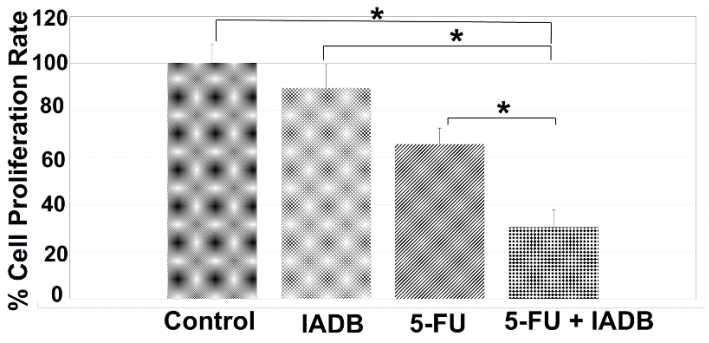

Figure S4. Effect of **IADB** on proliferation assay of HT-29 colon cancer cells. The effect of **IADB**-pretreatment on the inhibitory effect of 5-FU on HT-29 cell proliferation was investigated by trypan blue exclusion. Pretreatment of HT-29 cells with **IADB** at 10 μM for 12h prior to exposure to 5-FU (5 μM) for 48h resulted in significant inhibition of growth (66% *vs* 31% inhibition for 5-FU alone and **IADB** + 5-FU, respectively, *: $p<0.05$).

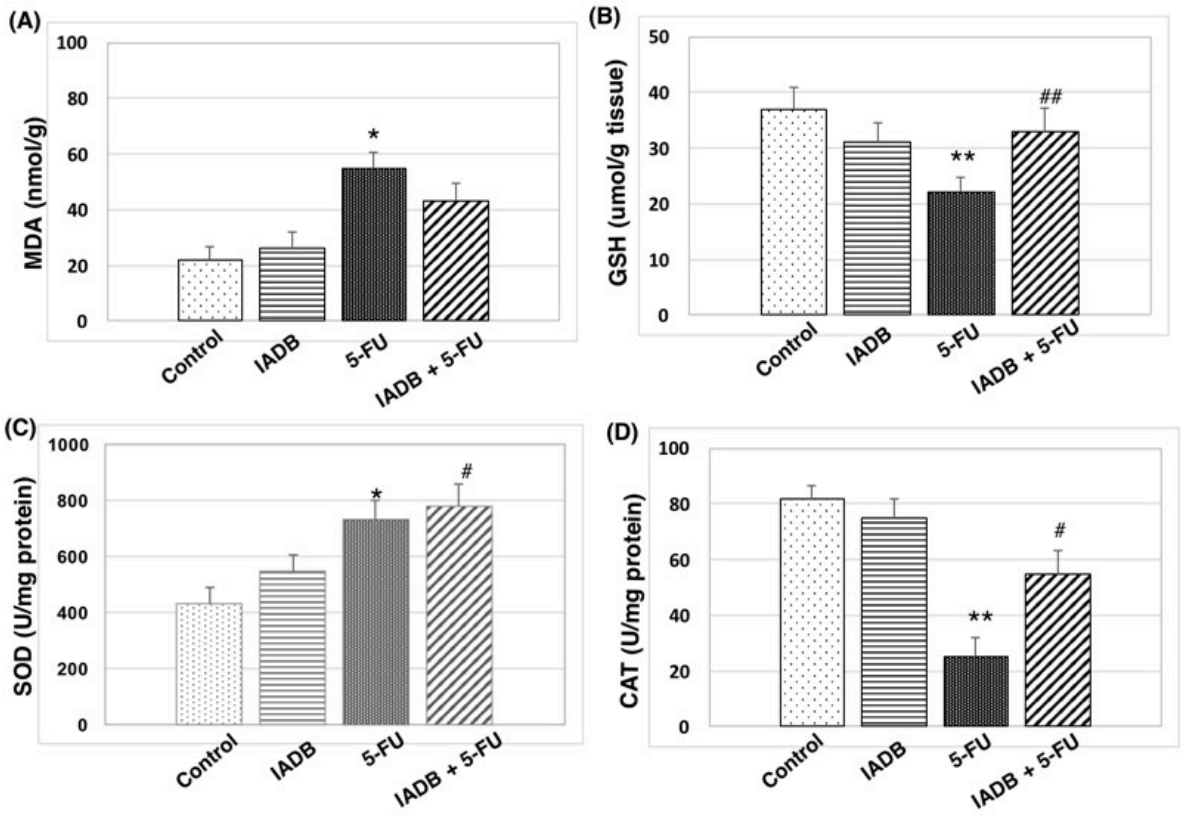

Figure S5. Evaluation of oxidative status of heart taken from sham control and different drug treatment groups: (**A**) Malondialdehyde (MDA) in homogenates of heart in sham-control and drug treatments (**IADB** alone; 5-FU alone; **IADB** +5-FU) rat cohorts; $^{*}p<0.05$: compared with sham control; (**B**) Glutathione (GSH) in homogenates of heart in sham control and drug treated cohorts (**IADB** alone; 5-FU alone; **IADB** +5-FU) ; $^{**}p<0.01$: compared with sham control; $^{\#\#}p<0.01$: compared with 5-FU alone ; (**C**) Superoxide dismutase (SOD) in heart homogenates of the same rat cohorts; $^{*}p<0.05$: compared with sham control; $^{\#}p<0.05$: compared with 5-FU alone; (**D**) Catalase (CAT) in heart homogenates in sham control and drug-treated cohorts (**IADB** alone; 5-FU alone; **IADB** +5-FU) ; $^{**}p<0.01$: compared with sham control; $^{\#}p<0.05$: compared with 5-FU alone.

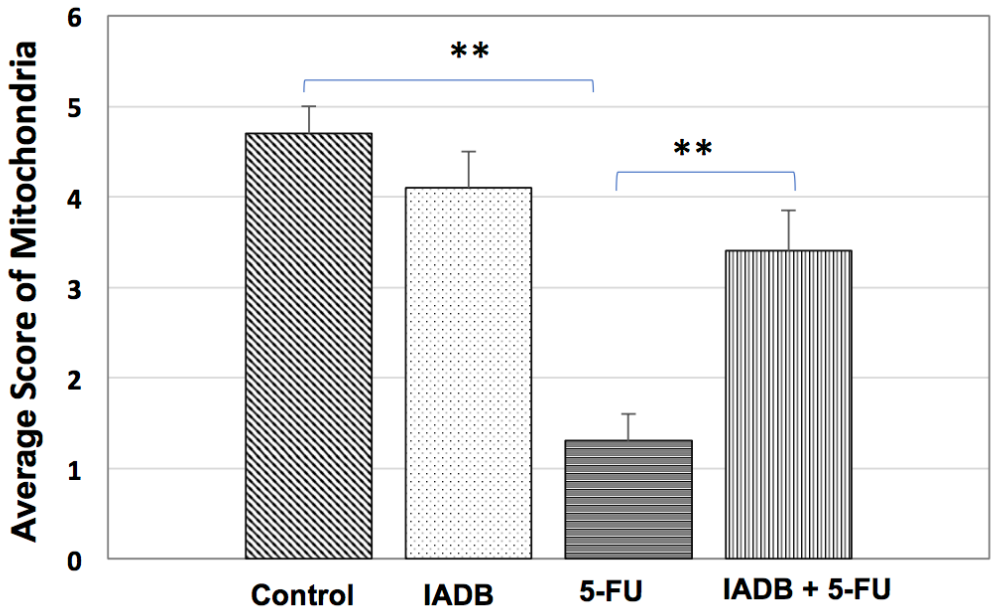

Figure S6. Transmission electron microscopy (TEM) scoring of mitochondria based on myocardium derived from sham control and different drug treatment group (IADB alone, 5-FU alone, and IADB + 5-FU combined treatment groups). Grade 5, intact mitochondria with normal cristae; Grade 4, slightly irregular mitochondria with occasional swollen cristae; Grade 3, irregular mitochondria with swollen cristae; Grade 2, fragmented mitochondria with swollen cristae and distorted membranes; Grade 1, severely damaged mitochondria with low density cristae and/or raptured inner and outer mitochondrial membrane.

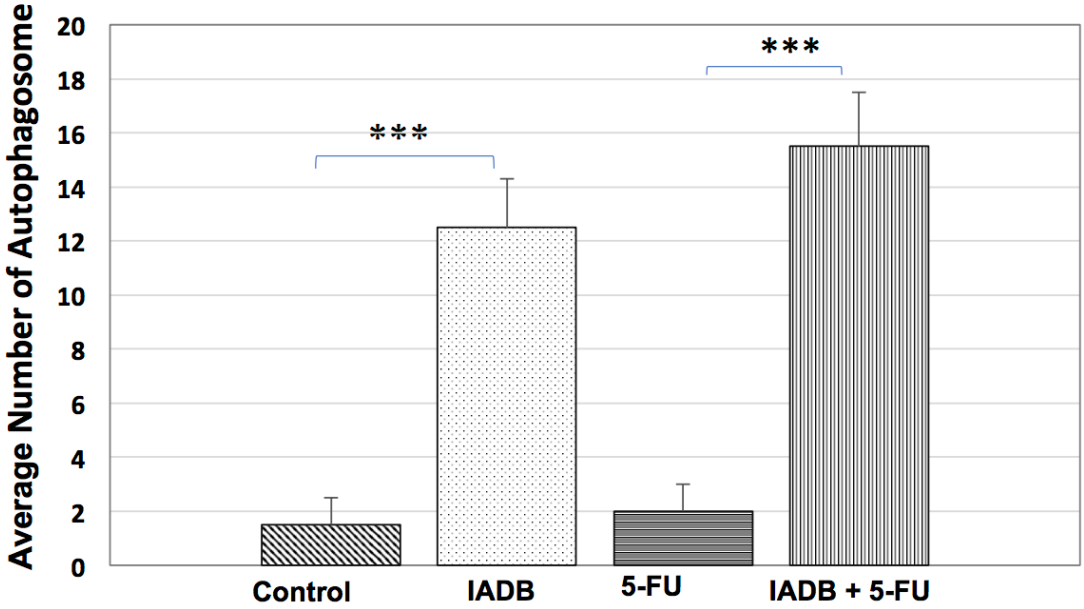

Figure S7. Transmission electron microscopy (TEM) analysis of autophagy based on myocardium derived from sham control and different drug treatment group (IADB alone, 5-FU alone, and IADB + 5-FU combined treatment groups). The average number of autophagosomes was counted in 10 fields under high resolution using TEM. ***P<0.001.