Supplementary Information

Low-dose HSP90 inhibitors DPB and AUY-922 repress apoptosis in HUVECs

Su-Yun Bai, [ac] Li-Qi Yao, [a] Le Su, [a] Shang-Li Zhang, [a] Bao-Xiang Zhao*[b] and Jun-Ying Miao*[a]

a Institute of Developmental Biology, School of Life Science, Shandong University, Jinan 250100, P.R.China.
b Institute of Organic Chemistry, School of Chemistry and Chemical Engineering, Shandong University, Jinan 250100, P.R. China.
c School of Basic Medical Sciences, Taishan Medical University, Taian 271000, P.R. China

Fax: +86 531 88565610

E-mail: miaojy@sdu.edu.cn, bxzhao@sdu.edu

SUPPLEMENTARY RESULTS

S1. Effects of low-dose DPB on the morphology and the viability of HUVECs

To evaluate the effects of low-dose DPB on serum withdrawal-induced apoptosis, the morphology of HUVECs was monitored under a phase contrast microscope. Serum withdrawal caused cells to become globular and detach from the bottom of culture dish indicating the occurrence of apoptosis. DPB (0.5-2 μM) decreased cell death and made HUVECs become elongated, which implied DPB might have the potential of apoptosis inhibition (**Figure S1b**). To further make certain the protective mechanism of DPB on HUVECs, SRB assay was performed. SRB assay showed that DPB (0.5-2 μM) significantly elevated endothelial cell (EC) viability (**Figure S1c**).

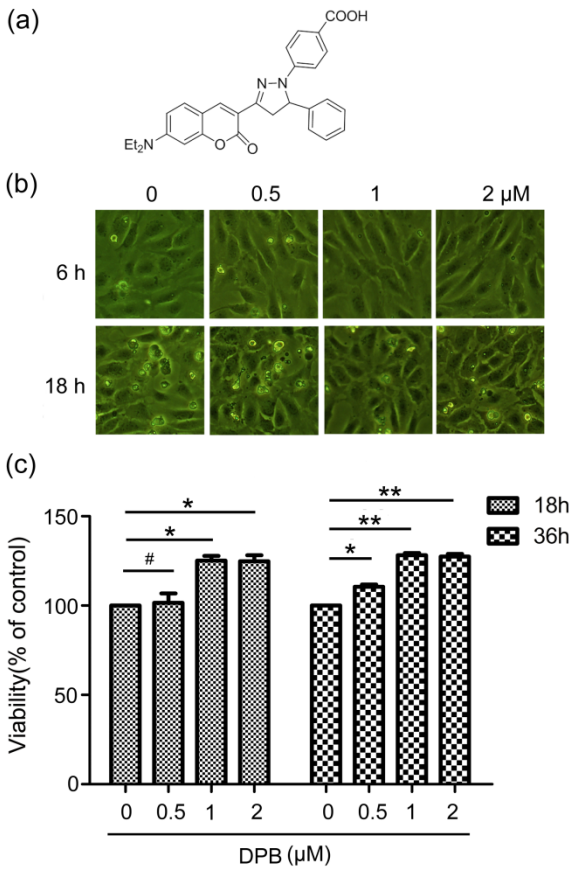

Fig. S1. Low-dose DPB protected HUVECs against serum deprivation-induced injury. **(a)** The chemical structure of DPB. **(b)** Morphology images of HUVECs cultured under serum

starvation in present or absent of DPB at indicated concentrations for 6 h or 18 h (200×). **(c)** Viability of HUVECs after treatment with DPB at indicated concentrations for 18 h or 36 h (#$p > 0.05$, *$p < 0.05$ and **$p < 0.01$; n = 3).

S2. High-dose DPB decreased the viability of HUVECs

Further studies on morphology (**Figure S2a**) and cell viability (**Figure S2b**) indicated that high-dose DPB（4-12 μM）increased the death of HUVECs and decreased the cell viability.

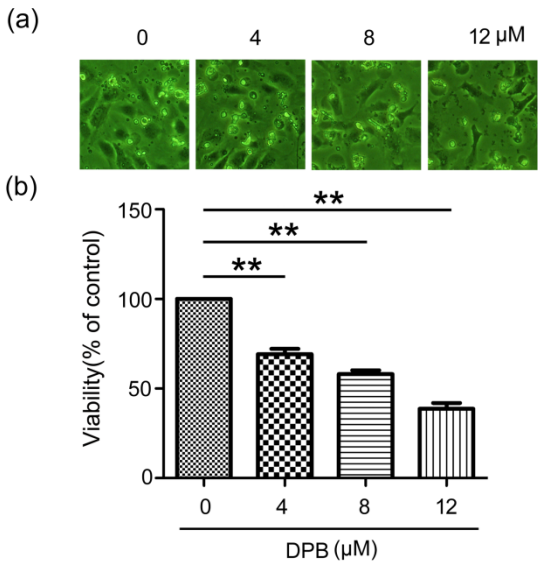

Fig. S2. High-dose DPB increased the death of HUVECs and decreased the cell viability. **(a)** Morphology images of HUVECs cultured under serum starvation in the presence or absence of DPB at indicated concentrations for 18 h (200×). **(b)** Viability of HUVECs after treatment with DPB at indicated concentrations for 18 h (**$p < 0.01$; n = 3).

S3. High-dose DPB promoted HUVECs apoptosis induced by serum deprivation

Hoechst 33258 staining (**Figure S3a**) and the detection of cleaved PARP (**Figure S3b**) showed high-dose DPB promoted HUVECs apoptosis induced by serum deprivation.

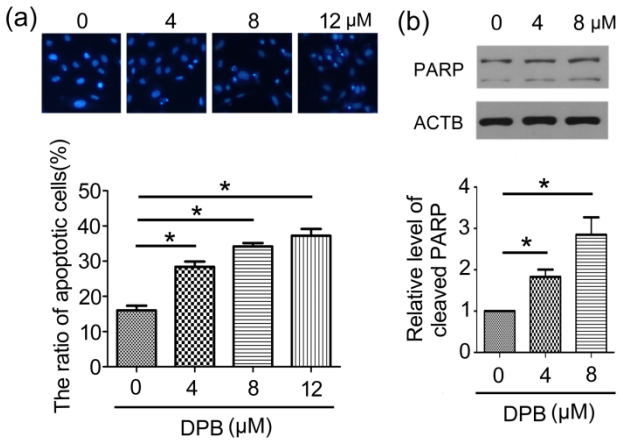

Fig. S3. High-dose DPB promoted HUVECs apoptosis caused by serum deprivation. **(a)** After treatment with indicated concentrations of DPB for 18 h, HUVECs deprived of serum were stained with Hoechst 33258 and the apoptosis rate was calculated. (*p < 0.05; n = 3). **(b)** After treatment with indicated concentrations of DPB for 18 h, the level of cleaved PARP was examined by Western blotting. (*p < 0.05; n = 3).

S4. Low-dose DPB did not affect the AKT and AKT1 levels in HUVECs

To investigate the effects of DPB on the levels of AKT and AKT1 in HUVECs, Western blotting was performed. The results indicated that low-dose DPB had no distinct disturbance on AKT and AKT1 levels (**Figure S4**).

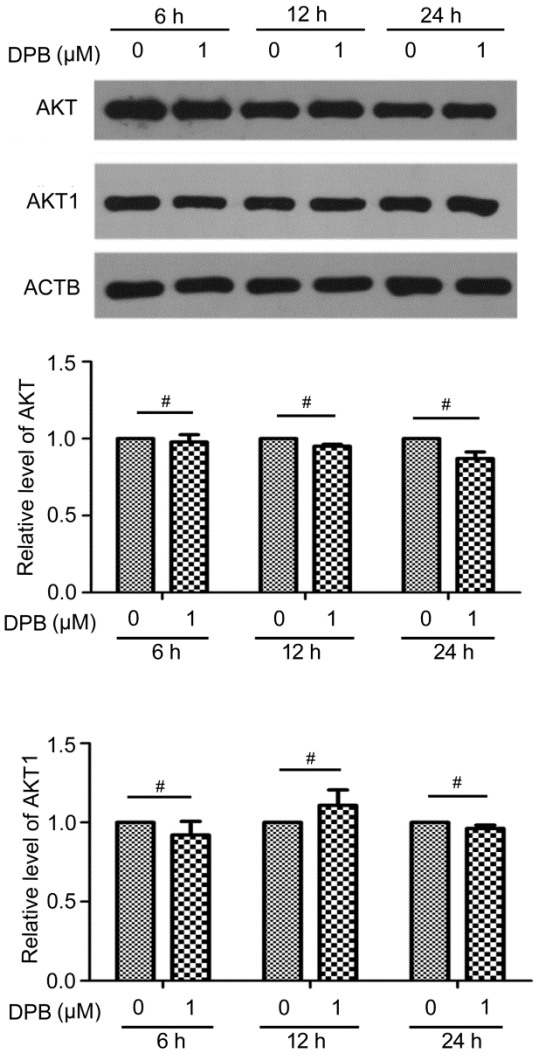

Fig.S4. Low-dose DPB had no distinct effect on the levels of AKT and AKT1. Western blotting analysis of AKT, AKT1 and ACTB proteins in HUVECs treated for different time with 1 μM of DPB. The graph represented the quantitation of AKT and AKT1 protein levels ($^{\#}p > 0.05$; n = 3).

S5. Low-dose DPB increased the level of p-AKT1 in the presence of VEGF

In many cancers, VEGF is a widely expressed glycoprotein playing critical roles in tumor angiogenesis. VEGF signaling regulating angiogenesis is mainly mediated by activation of its downstream signaling targets, including AKT pathway and others.[1] To investigate whether low-dose DPB affects the level of p-AKT1 in the presence of VEGF, after pretreatment with or without

DPB for 6 h, we stimulated serum starved HUVECs with 50 ng/mL of VEGF for 20 min. Then, the level of p-AKT1 was examined by Western blotting. The results showed that VEGF elevated the level of p-AKT1, and low-dose DPB pretreatment further increased the level of p-AKT1（**Figure S5**）.

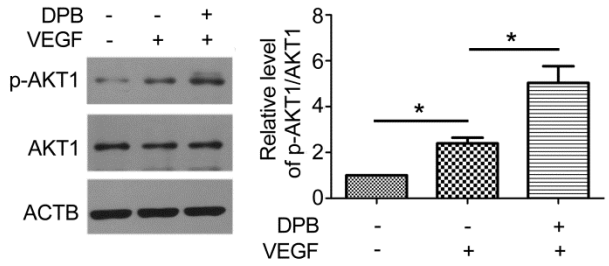

Fig.S5. Effect of low-dose DPB (1 μM) on the level of p-AKT1 in HUVECs in the presence of VEGF (*p < 0.05; n = 3).

S6. AUY-922 repressed the decline of p- AKT1 induced by serum starvation

To make certain whether other low-dose HSP90 inhibitors also have the function of repressing HUVECs apoptosis caused by serum starvation, the effects of NVP-AUY922 on HUVECs were investigated. The results indicated that NVP-AUY922 had similar effects to DPB. High-dose NVP-AUY922 (⩾5 nM) decreased HUVEC viability and low dose of NVP-AUY922 (5-20 pM) could significantly protect HUVECs from injury caused by serum starvation (**Figure S6**). Moreover, NVP-AUY922 could also activate AKT1 under serum starvation (**Figure S6b**).

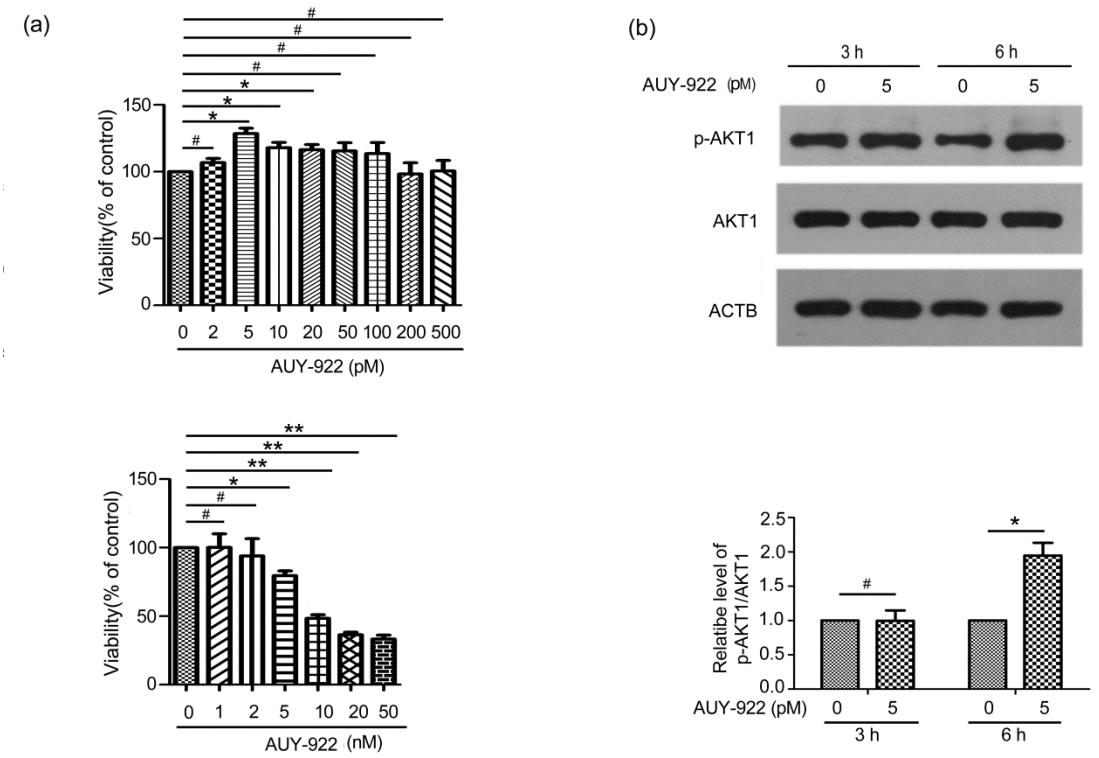

Fig.S6. Protective effects of low-dose AUY-922 on HUVECs against serum deprivation-induced injury. **(a)** Viability of HUVECs after treatment with indicated concentrations of AUY-922 for 24 h ($^{\#}p > 0.05$, $^{*}p < 0.05$ and $^{**}p < 0.01$; n = 3). **(b)** Western blot analysis of p-AKT1 in HUVECs treated for different time with 5 pM AUY-922 ($^{\#}p > 0.05$ and $^{*}p < 0.05$; n = 3).

EXPERIMENTAL SECTION

Cell culture and exposure to DPB or AUY-922

HUVECs were obtained by the method of Jaffe et al.[2] HUVECs were cultured in M199 medium (Gibco Laboratories, Grand Island, NY) with 10% fetal bovine serum (Hyclone, SV30087.02) and 2 ng/ml basic fibroblast growth factor (bFGF) at 37 ℃ with 5% CO_2. Passage number of HUVECs used in the experiments was less than 10.

After reached sub-confluence, the cells were washed once with basal M199 medium (without serum and bFGF) and cultured in basal M199 medium containing DPB or AUY-922. Dimethyl sulfoxide (DMSO) was used as the vehicle control. The final concentration of DMSO is less than 0.1% (v/v) in the culture medium.

Cell viability assay

Cells were seeded onto 96-well plates and treated with DPB or AUY-922 at different concentration for appropriate time. SRB assay was used to determine the cell viability. Each experiment was conducted in triplicate.

Hoechst 33258 staining

Cells were seeded in 24-well plates before treatment with DPB. Eighteen hours after treatment, the cells were stained with 10 μg/ml Hoechst 33258 (sigma) for 10 min at 37 ℃, then gently washed once with PBS and photographed by an Olympus (Japan) BH-2 fluorescence microscope. For the calculation of apoptosis rate, a minimum of 500 cells was counted, and the results were from triplicate assays.

Antibodies

Antibodies for PARP (9542L), AKT (9272), and p-AKT1 (9018) were from Cell Signaling Technology (Danvers, MA, USA); β-ACTIN (sc-47778), AKT1 (sc-1618) and horseradish peroxidase-conjugated secondary antibodies were all from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Western blotting analysis

Whole cell lysates were prepared in western and IP lysis buffer. Protein samples were separated by SDS-PAGE and transferred to a polyvinylidenedifluoride (PVDF) membrane (Millipore,

IPVH00010). After incubated with primary antibodies (1:1000), the membrane was further incubated with peroxidase-conjugated secondary antibodies (1:5000), and then detected by an enhanced chemiluminesence detection kit (Thermo Fisher, 34080). The relative quantity of proteins was analyzed by Quality One software.

Migration Assay

Cell migration assay was performed as described by Ryan et al.[3] Briefly, cells were seeded on pretraced 6-well plates, and a portion of the cell monolayer was scraped away with a sterile pipette tip. After washed with medium twice, cells were incubated with DPB over 6, 12, and 24 h. Cell migration was quantified by measuring the distance between the wound edges before and after injury with the use of ImageJ software.

Statistical analyses

Data were analyzed by SPSS 13.0 (Statistical Package for the Social Sciences) and expressed as mean ± SEM. Results from at least 3 independent experiments were analyzed. *P*-value of < 0.05 was considered statistically significant.

Reference List

1. I. Zachary and G. Gliki, *Cardiovascular research*, 2001, **49**, 568-581.
2. E. A. Jaffe, R. L. Nachman, C. G. Becker and C. R. Minick, *The Journal of clinical investigation*, 1973, **52**, 2745-2756.
3. U. S. Ryan, M. Absher, B. M. Olazabal, L. M. Brown and J. W. Ryan, *Tissue & cell*, 1982, **14**, 637-649.