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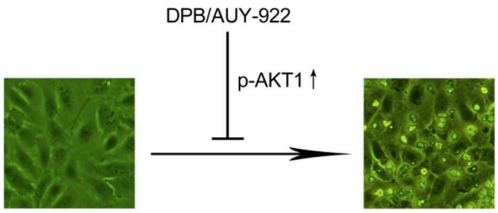

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Low-dose HSP90 inhibitors DPB and AUY-922 repress apoptosis in HUVECs †

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In this study, we found that low-dose HSP90 inhibitors DPB and AUY-922 could unexpectedly restrain apoptosis in HUVECs. This hormesis was accompanied by the increase of p-AKT1. Our findings could have significant implications for the administration of HSP90 inhibitors in vascular diseases and cancer.

Endothelial injury can result in the occurrence and development of many vascular diseases including atherosclerosis, thrombus formation and plaque erosion.[1] As is well-known, deprivation of serum and growth factor to mimic ischemia induces apoptosis of endothelial cells (ECs). Therefore, restraining endothelial apoptosis caused by serum and growth factor deprivation is of great significance for vascular disease prevention and treatment. However, drugs developed for this purpose are still in deficiency. Our previous study revealed that 50-200 μM 6-amino-2,3-dihydro-3-hydroxymethyl-1,4-benzoxazine (ABO) could prevent human umbilical vein endothelial cells (HUVECs) death induced by serum and growth factor deprivation.[2] But the minimum needed concentration of ABO is very high for apoptosis repression.

Heat shock protein 90 (HSP90) is an ubiquitously expressed molecular chaperone which is involved in the folding, activation and assembly of its client proteins.[3] Currently, more than 200 client proteins have been found and many of them are involved in multiple oncogenic processes.[4] Therefore, HSP90 inhibitors are emerging as novel promising therapeutic agents for cancer therapy and more than 17 HSP90 inhibitors have entered clinical trials.[4-7] But HSP90 inhibitors have shown clear clinical activity in only a handful of studies.[3] The reason for this lack of efficacy is currently not clear.

Tumor vascularization is an essential modulator of early tumor growth, progression, and therapeutic effect. Recent studies suggest that many HSP90 inhibitors show anti-angiogenic properties by disrupting the PI-3K/AKT/eNOS signal transduction pathway in ECs, as well as through decreasing the expression of VEGFR-2, a crucial component of the angiogenic process.[8]

Here we present evidence for a mechanism that may compromise the efficacy of HSP90 inhibitors. We show that, to our surprise, low dose of HSP90 inhibitors DPB (Fig. S1a, ESI†) and AUY-922 could repress apoptosis in HUVECs. This hormesis was accompanied by the increase of p-AKT1. This may compromise the anticancer activity of HSP90 inhibitors and may provide an explanation as to why these agents are usually ineffective in clinical trials. Moreover, low-dose HSP90 inhibitors might have a promising application in the therapy of vascular diseases caused by ischemia.

4-(3-(7-(diethylamino)-2-oxo-2H-chromen-3-yl)-5-phenyl-4,5-dihydro-1H-pyrazol-1-yl)benzoic acid (DPB) was a new HSP90

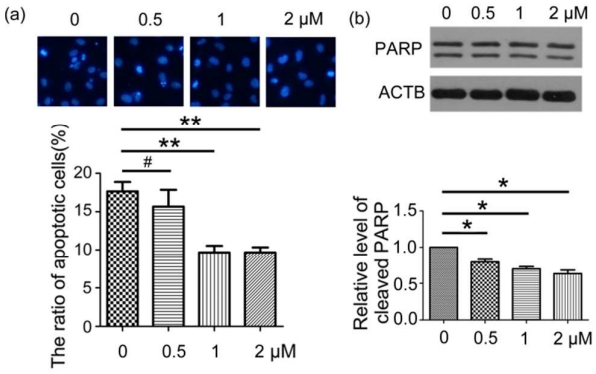

Fig. 1 Low-dose DPB suppressed HUVECs apoptosis caused by serum deprivation. (a) After treatment with indicated concentrations of DPB for 18h, serum deprived HUVECs were stained with Hoechst 33258 and the apoptosis rate was calculated. (**$p < 0.01$ and $^{\#}p > 0.05$; n = 3). (b) After treatment with indicated concentrations of DPB for 18h, the level of cleaved PARP was examined by Western blotting. (*$p < 0.05$; n = 3).

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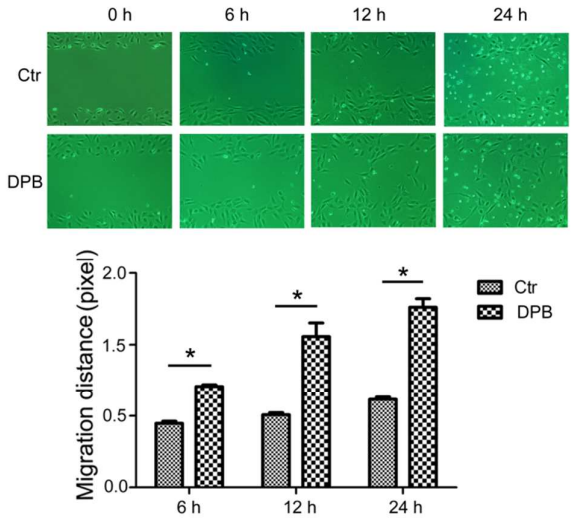

Fig.2 Low-dose DPB (1 μM) promoted HUVECs migration in the absence of serum and bFGF in a time-dependent manner. HUVECs were incubated with DPB over 6, 12, and 24 h, and cell migration was tested by scratched wound assay. Representative images of cell migration after different time of incubation with DPB were shown. Data are mean ± SEM (*$p < 0.05$ vs control; n = 3).

Inhibitor developed by us recently.[9] Our previous study showed that DPB (5-20 μM) could induce apoptosis in A549 lung carcinoma cells. To evaluate the effects of DPB on serum withdrawal-induced apoptosis, the morphology study (Fig. S1b and Fig. S2a, ESI†) and viability assay (Fig. S1c and Fig. S2b, ESI†) of HUVECs were performed. Results showed low-dose DPB (0.5-2 μM) might have the potential of apoptosis inhibition and high-dose DPB (4-12 μM) might promote apoptosis. To further make certain the action mechanism of DPB on HUVECs, Hoechst 33258 staining and the detection of cleaved PARP were performed. Results showed that low-dose DPB could inhibit apoptosis in HUVECs (Fig. 1a and Fig. 1b) and high-dose DPB had the opposite effect (Fig. S3a and Fig. S3b, ESI†).

Endothelial survival pathway can promote angiogenesis. Endothelial cell migration is essential to angiogenesis. To detect the effect of low-dose DPB on HUVECs migration, scratch wound assay was performed. As shown in Fig. 2, DPB at the concentration of 1 μM increased ECs migration in a time-dependent manner.

AKT is an important HSP90 client protein. AKT has been implicated as an anti-apoptotic mediator in numerous cell death paradigms, including withdrawal of extracellular matrix, oxidative and osmotic stress, ischemic shock, irradiation and treatment of cells with chemotherapeutic agents.[10] Our previous study showed that high-dose DPB (20 μM) could induce the apoptosis of A549 lung carcinoma cells by decreasing the levels of AKT and p-AKT.[9] In ECs, AKT can promote phosphatidylinositol 3-kinase (PI 3-kinase)-dependent endothelial cell (EC) survival[11, 12] and migration. Since low-dose DPB could restrain endothelial apoptosis and promote migration, we investigated whether low-dose DPB could affect the AKT level in ECs. The results indicated that 1 μM DPB had no distinct effect on AKT levels (Fig. S4, ESI†). The AKT kinase family contains three different isoforms: AKT1 (PKBα), AKT2 (PKBβ) and AKT3 (PKBγ).[13] Among these isoforms, AKT1 is predominantly expressed in ECs.[14] Several studies have demonstrated the important roles of AKT1 in ECs. AKT1 is a critical downstream kinase in the vascular endothelial growth factor (VEGF) signalling cascade[15] and cultured AKT1 $^{-/-}$ ECs exhibit impaired NO release, integrin activation, migration, and proliferation.[14, 16, 17] Phosphorylation of AKT1 will greatly increase its activity. The effect of serum starvation on the level of p-AKT1 has not been reported. Our study showed the level of p-AKT1 was decreased after serum starvation (Fig. 3). Interestingly, further study showed that although DPB did not disturb the level of AKT1 (Fig. S4, ESI†), it could significantly repress the decline of p-AKT1 caused by serum starvation (Fig. 3). And DPB could increase p-AKT1 in the presence of VEGF (Fig. S5, ESI†). What's more, low dose of NVP-AUY922 (5-20 pM), another well-known HSP90 inhibitor could also significantly protect HUVECs from injury caused by serum starvation (Fig.S6a, ESI†). NVP-AUY922 could also restrain the decline of p-AKT1 caused by serum starvation (Fig. S6b, ESI†). It was reported that low-dose HSP90 inhibitor 17-allylamino-17-demethoxygeldanamycin (17-AAG) prevented neural progenitor cells from either naturally-occurring or stress-induced apoptosis by activating multiple pro-survival factors including AKT.[18] These studies indicated that low dose of HSP90 inhibitors might have the general function of repressing HUVECs apoptosis caused by serum starvation.

Due to its important roles in regulating the stability, activity and intracellular sorting of oncogenic client proteins, HSP90 inhibitors have already become one of the most promising cancer treatment targets. In cancer cells, client proteins of HSP90 play important roles in carcinogenic signal transduction (such as mutative EGFR),

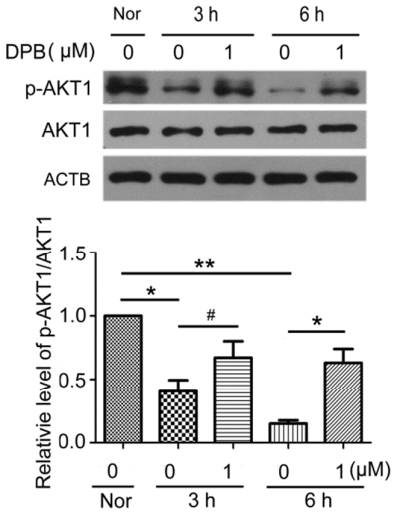

Fig. 3 Effects of serum deprivation and low-dose DPB (1 μM) on the level of p-AKT1 in HUVECs (*$p < 0.05$, **$p < 0.01$ and $^{\#}p > 0.05$; n = 3).

angiogenesis (like VEGF), anti-apoptosis (such as AKT) and metastasis (like MMP2 and CD91).[4] So far, a variety of HSP90 inhibitors have been discovered and developed, and some of them have entered clinical trials. But HSP90 inhibitors have shown clear clinical efficacy in only a handful of studies. The reason is currently unclear. Some compounds that suppress cell growth at high concentrations can promote cell growth at lower concentrations-that is, their dose-response curve is 'bell-shaped'.[19, 20] The Low dose stimulation and high dose inhibition phenomenon, namely biphasic dose response is called hormesis in the field of toxicology. [19, 21] Hormesis has currently become a central concept in biological and biomedical sciences with significant implications for clinical medicine. Many researches attempted to make certain mechanisms accounting for the hormesis. On the whole, no single "hormetic" mechanism can explain the plethora of such biphasic concentration. Different cell type and agent appears to have a unique action mechanism.[19, 20, 22] Presently, researches on the hormesis of HSP90 inhibitors are very few. In this study, we show that low dose of HSP90 inhibitors DPB and AUY-922 unexpectedly restrain HUVECs apoptosis and promote migration by repressing the decline of p-AKT1 caused by serum starvation. This mechanism may be an important reason that HSP90 inhibitors often have poor efficacy. So the dosing and administration of HSP90 inhibitors in the clinical trial should be reevaluated. Our results implied that for HSP90 inhibitors therapy it would be favourable to maintain high plasma concentrations and to avoid low circulating concentrations. Alternatively, this problem could be overcome by combining HSP90 inhibitors with anti-angiogenesis therapy including agents that could inhibit the phosphorylation of AKT1. Furthermore, our work indicated low-dose HSP90 inhibitors could be potential agents in improving endothelial function and treatment of apoptosis related cardiovascular diseases.

Acknowledgements

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Notes and references

1. H. Kobayashi, N. Ouchi, S. Kihara, K. Walsh, M. Kumada, Y. Abe, T. Funahashi and Y. Matsuzawa, *Circulation research*, 2004, **94**, e27-31.
2. P.-F. Jiao, B.-X. Zhao, W.-W. Wang, Q.-X. He, M.-S. Wan, D.-S. Shin and J.-Y. Miao, *Bioorganic & medicinal chemistry letters*, 2006, **16**, 2862-2867.
3. S. Soga, S. Akinaga and Y. Shiotsu, *Current pharmaceutical design*, 2013, **19**, 366-376.
4. D. S. Hong, U. Banerji, B. Tavana, G. C. George, J. Aaron and R. Kurzrock, *Cancer treatment reviews*, 2013, **39**, 375-387.
5. Y. S. Kim, S. V. Alarcon, S. Lee, M. J. Lee, G. Giaccone, L. Neckers and J. B. Trepel, *Current topics in medicinal chemistry*, 2009, **9**, 1479-1492.
6. K. Jhaveri, T. Taldone, S. Modi and G. Chiosis, *Biochimica et biophysica acta*, 2012, **1823**, 742-755.
7. L. Neckers and P. Workman, *Clinical cancer research : an official journal of the American Association for Cancer Research*, 2012, **18**, 64-76.
8. K. Staufer and O. Stoeltzing, *Current cancer drug targets*, 2010, **10**, 890-897.
9. S. Y. Bai, X. Dai, B. X. Zhao and J. Y. Miao, *Rsc Adv*, 2014, **4**, 19887-19890.
10. P. R. Somanath, O. V. Razorenova, J. Chen and T. V. Byzova, *Cell cycle*, 2006, **5**, 512-518.
11. H.-P. Gerber, A. McMurtrey, J. Kowalski, M. Yan, B. A. Keyt, V. Dixit and N. Ferrara, *Journal of Biological Chemistry*, 1998, **273**, 30336-30343.
12. A. Papapetropoulos, D. Fulton, K. Mahboubi, R. G. Kalb, D. S. O'Connor, F. Li, D. C. Altieri and W. C. Sessa, *The Journal of biological chemistry*, 2000, **275**, 9102-9105.
13. E. Gonzalez and T. E. McGraw, *Cell cycle*, 2009, **8**, 2502-2508.
14. J. Chen, P. R. Somanath, O. Razorenova, W. S. Chen, N. Hay, P. Bornstein and T. V. Byzova, *Nature medicine*, 2005, **11**, 1188-1196.
15. G. Zhuang, K. Yu, Z. Jiang, A. Chung, J. Yao, C. Ha, K. Toy, R. Soriano, B. Haley, E. Blackwood, D. Sampath, C. Bais, J. R. Lill and N. Ferrara, *Science signaling*, 2013, **6**, ra25.
16. P. R. Somanath, J. Chen and T. V. Byzova, *Angiogenesis*, 2008, **11**, 277-288.
17. M. Y. Lee, A. K. Luciano, E. Ackah, J. Rodriguez-Vita, T. A. Bancroft, A. Eichmann, M. Simons, T. R. Kyriakides, M. Morales-Ruiz and W. C. Sessa, *Proceedings of the National Academy of Sciences of the United States of America*, 2014, **111**, 12865-12870.
18. G. Wang, K. Krishnamurthy and D. Tangpisuthipongsa, *Journal of neurochemistry*, 2011, **117**, 703-711.
19. E. J. Calabrese, *Critical reviews in toxicology*, 2005, **35**, 463-582.
20. A. R. Reynolds, I. R. Hart, A. R. Watson, J. C. Welti, R. G. Silva, S. D. Robinson, G. Da Violante, M. Gourlaouen, M. Salih, M. C. Jones, D. T. Jones, G. Saunders, V. Kostourou, F. Perron-Sierra, J. C. Norman, G. C. Tucker and K. M. Hodivala-Dilke, *Nature medicine*, 2009, **15**, 392-400.
21. E. J. Calabrese, *Homeopathy : the journal of the Faculty of Homeopathy*, 2015, **104**, 69-82.
22. A. Skaletz-Rorowski and K. Walsh, *Current opinion in lipidology*, 2003, **14**, 599-603.