Dehydrocostus lactone Suppresses Angiogenesis In Vitro and In Vivo through Inhibition of Akt/GSK-3β and mTOR Signaling Pathways

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Abstract

The traditional Chinese medicine component dehydrocostus lactone (DHC) isolated from Saussurea costus (Falc.) Lipschitz, has been shown to have anti-cancer activity. Angiogenesis is an essential process in the growth and progression of cancer. In this study, we demonstrated, for the first time, the anti-angiogenic mechanism of action of DHC to be via the induction of cell cycle progression at the G0/G1 phase due to abrogation of the Akt/glycogen synthase kinase-3β (GSK-3β)/cyclin D1 and mTOR signaling pathway. First, we demonstrated that DHC has an anti-angiogenic effect in the matrigel-plug nude mice model and an inhibitory effect on human umbilical vein endothelial cell (HUVEC) proliferation and capillary-like tube formation in vitro. DHC caused G0/G1 cell cycle arrest, which was associated with the down-regulation of cyclin D1 expression, leading to the suppression of retinoblastoma protein phosphorylation and subsequent inhibition of cyclin A and cdk2 expression. With respect to the molecular mechanisms underlying the DHC-induced cyclin D1 down-regulation, this study demonstrated that DHC significantly inhibits Akt expression, resulting in the suppression of GSK-3β phosphorylation and mTOR expression. These effects are capable of regulating cyclin D1 degradation, but they were significantly reversed by constitutively active myristoylated (myr)-Akt. Furthermore, the abrogation of tube formation induced by DHC was also reversed by overexpression of Akt. And the co-treatment with LiCl and DHC significantly reversed the growth inhibition induced by DHC. Taken together, our study has identified Akt/GSK-3β and mTOR as important targets of DHC and has thus highlighted its potential application in angiogenesis-related diseases, such as cancer.

Introduction

Angiogenesis, which is the process of formation of new blood vessels from pre-existing ones, takes place throughout physiological development, tissue repair, and reproduction [1]. In pathological conditions, angiogenesis is also essential for tumor growth and progression to ensure that more oxygen and nutrients are delivered from the host’s vascular system. The induction of tumor vasculatures, termed the angiogenic switch, is initiated by various growth factors (vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) are the most commonly ubiquitous) released from tumor cells, and is tightly regulated by the balance of angiogenic activators and inhibitors [2]. The angiogenic factors stimulate the degradation of extracellular matrix components of the parent vessels, which allow endothelial cells to migrate, proliferate, and form tube-like structures. After forming new capillary sprouts, the blood vessels mature. Thus, inhibition of the steps of angiogenesis by blocking angiogenesis-related proteins could be a strategy to arrest tumor growth [3].

Akt is a serine/threonine protein kinase activated by growth factors, which transmits survival signals to downstream effectors. In endothelial cells, the major growth factor-induced angiogenic responses are mediated predominantly by Akt signaling [4]. Activation of the Akt signaling pathway is critical for cell proliferation and growth of endothelial cells. After receptor stimulation, Akt targets a number of established substrates for phosphorylation. One of the major effectors downstream of Akt is glycogen synthase kinase (GSK)-3β [5,6]. Upon the activation of Akt, GSK-3β inactivates GSK-3β by initiating phosphorylation by its own serine 9 residue. GSK-3β is thought to regulate cell cycle progression by phosphorylating cyclin D1 at the threonine 286 residue. This later promotes nuclear export and targets the protein for ubiquitylation and subsequent degradation by proteasomes [7,8]. Therefore, growth factors promote cyclin D1 stabilization and up-regulation through the Akt/GSK-3β pathway [9]. Cyclin
DHC is a potential anti-angiogenic agent. GSK-3β demonstrated that DHC inhibits angiogenesis through the Akt/mTOR pathways. Furthermore, the in vitro matrigel plug assay indicated that DHC is a potential anti-angiogenic agent.

**Results**

**Effect of DHC on angiogenesis in vivo**

To determine the role of DHC in the regulation of angiogenesis in vivo, we analyzed matrigel plug formation following subcutaneous implantation in mice. Matrigel mixed with angiogenic growth factors induced blood vessel growth and showed blood cell-filled angiogenic vasculature. In contrast, DHC significantly inhibited neo-vascularization in a concentration-dependent manner (Figure 1A). Following Masson's trichrome staining, the vasculature within the plugs identified more vessel-like structures within the control group than in the DHC-treated groups. This study then quantified the vascular formation in the matrigel by analyzing the hemoglobin content. The plugs mixed with a variant of growth factors contained 117.3 mg/mL of hemoglobin, identical to the formation of functional vasculature in the matrigel (Figure 1B). Co-treatment with DHC reduced the hemoglobin content in the plugs in a concentration-dependent manner (Figure 1A). Following Masson's trichrome staining, the vasculature within the plugs identified more vessel-like structures within the control group than in the DHC-treated groups. This study then quantified the vascular formation in the matrigel by analyzing the hemoglobin content. The plugs mixed with a variant of growth factors contained 117.3 mg/mL of hemoglobin, identical to the formation of functional vasculature in the matrigel (Figure 1B). Co-treatment with DHC reduced the hemoglobin content in the plugs in a concentration-dependent manner, demonstrating that DHC has potential inhibitory activity in growth factors-induced angiogenesis in vivo.

**Effect of DHC on angiogenesis in vitro**

In angiogenic processes, endothelial cells must undergo ECM degradation, migration, proliferation, and tube formation to form new blood vessels [2]. To investigate whether DHC inhibits angiogenesis through these steps in vitro, we transfected HUVECs with the constitutively active forms of Akt and GSK-3β and MTOR pathways. Furthermore, the in vitro matrigel plug assay indicated that DHC is a potential anti-angiogenic agent.

DHC causes G0/G1 phase cell cycle arrest in endothelial cells

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**Down-regulation of cyclin D1 by DHC causes growth inhibition via the Akt/GSK-3β pathway**

Previous studies have shown that the serine/threonine kinase Akt signals in response to a variety of growth factors, including platelet-derived growth factor (PDGF), bFGF [24,25], and VEGF [26]. To further delineate the mechanism that underlies the anti-angiogenic effect of DHC, the effect of DHC on Akt activation was examined by immunoblot analysis. Treatment with DHC dramatically suppressed EGM-2–induced Akt phosphorylation in the time- and concentration-dependent manners (Figure 5A). To verify that Akt was responsible for the proliferative response and to regulate the expression of the cell cycle protein cyclin D1, we transiently transfected HUVECs with the constitutively active form of Akt and then determined its effect on DHC-induced growth inhibition. Transfection efficiency was confirmed, as shown in Figure 5B. LY294002– (an Akt inhibitor, used as a positive control) and DHC-treated HUVECs over-expressing Akt exhibited a highly significant increase in the expression of cyclin D1 relative to the vector-treated group (Figure 5C).

Akt signaling negatively regulates GSK-3β activity by phosphorylating Ser-9, and GSK-3β was shown to regulate the cell cycles through cyclin D1 protosome-mediated degradation [8]. This study investigated whether the inhibition of Akt phosphorylation by DHC led to a decrease in GSK-3β phosphorylation levels and consequently contributed to growth inhibition. As shown in Figure 5D, treatment with DHC inhibited GSK-3β phosphorylation in the concentration- and time-dependent manners. However, DHC-treated cells combined with the over-expression of Akt increased the phosphorylation of GSK-3β compared with the vector group. As determined using the crystal
violet assay, the growth inhibition induced by DHC was found to be reversed by myristoylated (myr)-Akt treatment (Figure 6A). Over-expression of Akt in HUVECs also partially reversed the abrogation of tube formation induced by DHC (Figure 6B). To verify whether GSK-3β is involved as a downstream effector of Akt in the anti-angiogenic process of DHC, we treated HUVECs with LiCl, a GSK-3β activity inhibitor. Co-treatment of the LiCl with DHC significantly reversed the growth inhibition by approximately 50% of the DHC-treated cells (Figure 6C). Taken together, these results indicated that inhibition of the Akt/GSK-3β/cyclin D1 pathway plays an important role in DHC-induced growth inhibition and abrogation of tube formation in HUVECs.

**DHC suppresses expression of cyclin D1 in an mTOR-dependent manner**

Akt signaling is required for growth factor-induced activation of mammalian target of rapamycin (mTOR)-G1, and the activity of mTORC1 is impaired in Akt-deficient cells [27,28]. The activation of mTORC1 also leads to the phosphorylation of 4-epamil-binding protein (eBP)-1 and, therefore, activates eIF4E to up-regulate the translational levels of cyclin D1 [29]. To evaluate the contribution of mTORC1 in the DHC-induced downregulation of cyclin D1 in HUVECs, the downstream signaling effectors of mTORC1 were examined. We found that DHC effectively suppressed EGM-2-induced phosphorylation of the mTOR signaling cascade, including mTOR, p70S6K, 4EBP, and eIF4E, in a time-dependent manner (Figure 7A). DHC also inhibited the phosphorylation of mTOR downstream molecules in a concentration-dependent manner (Figure 7A). In HUVECs overexpressing Akt, the inhibitory effects of DHC on the phosphorylation of mTOR downstream signaling molecules had significantly reversed protein expression relative to the vector group (Figure 7B). Using the crystal violet assay, the co-treatment of rapamycin and DHC increased the growth inhibition effect...
compared with DHC alone (Figure S3). These results indicated that the Akt/mTOR signaling pathway could be involved in the growth arrest induced by DHC inhibition of cyclin D1.

**Discussion**

Tumor progression and metastatic shedding are dependent on angiogenesis [30]. Thus, in recent years, the development of anti-angiogenic agents has become attractive. Additionally, natural products have been reported to have a cytotoxic effect on the normal cell cycle. However, thus far, out of 22 anti-angiogenic agents, there are only 11 natural products or natural modified compounds have been under clinical trials [31]. This study found that DHC, a component of traditional Chinese medicine, has anti-angiogenic effects in vivo and in vitro. First, this study utilized the matrigel plug assay, which provided strong evidence that DHC inhibited angiogenesis in vivo in a dose-dependent manner. Previous studies have revealed that DHC suppresses the growth of various human tumors in xenograft mouse models [21,22,23]. Therefore, the inhibition of angiogenesis by DHC seems to be one of the main mechanisms that may hinder tumor progression. A series of in vitro assays established that the inhibition of EGM-2-induced tube formation and endothelial cell proliferation were consistent with this study’s in vivo result. In contrast, DHC had no significant inhibitory effect on cell migration in the transwell assay. Using the western blot, activation of p38 and ERK1/2 signaling has been observed when HUVECs were treated with DHC (figure S1B). We employed another system, a scratch wound healing assay to double confirm the effect of DHC on HUVECs migration. However, only combined treatment with p38 and DHC significantly inhibited HUVECs migration (figure S1A). The result indicated that even with inhibition of PI3K/Akt pathways, p38

![Figure 2. Impairment of in vitro angiogenesis by DHC.](image)

**Figure 2. Impairment of in vitro angiogenesis by DHC.** A, HUVECs were treated with or without DHC (0.3–10 μM) in EGM-2 medium. After 72 h of incubation, cells were stained with crystal violet and determined the inhibition of cell proliferation by the absorbance at 550 nm. B, DNA synthesis was assessed by BrdU incorporation assay. C, representative photographs of capillary-like structures formation of CTL and DHC-treated HUVECs on matrigel under microscope (magnification is X100). D, Quantification of the total tube length of capillary-like structures by image analysis software. Data represent the mean ± SEM from three independent experiments. ** P<0.01 and *** P<0.001 versus control. E, effect of DHC on cell migration using a transwell assay. F, the graph shows quantitative analysis of the migrated cell numbers in tranwell assay. Data represent the mean ± SEM from three independent experiments. ## P<0.01 versus basal.

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![Figure 3. Effect of DHC on cell cycle.](image)

**Figure 3. Effect of DHC on cell cycle.** A, HUVECs were starved for 24 h, and then were treated with or without DHC (3–10 μM) for 18 hr. After cells were labeled with propidium iodide, DNA content was analyzed by flow cytometry. B and C, cell population in G0/G1 and S/G2/M phase were quantified. Data represent the mean ± SEM from four independent experiments. * P<0.05, ** P<0.01 and *** P<0.001 versus control.

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activation contributed to the signaling of HUVECs migration while Akt signaling is inhibited. Taken together, we suggested that DHC blocked blood vessel formation by the selective inhibition of endothelial cell growth and capillary-like structure formation.

Cell proliferation is a critical event in the process of angiogenesis. This study found that DHC delayed the transition of HUVECs from the G0/G1 phase to the S phase. These results are consistent with those of a previous study, which found that DHC treatment induced growth inhibition in breast cancer [23]. Cyclin D1, known as an essential mitogenic signal sensor and cell cycle regulator, binds to CDK4 and forces cells to enter the proliferative stage of cell cycle from the G0 phase. Cyclin D1 reportedly plays an important role in endothelial cells. More recent studies have demonstrated that overexpression of cyclin D1 is linked to the development of various cancers [12]. Therefore, strategies to down-regulate cyclin D1 expression were reported to inhibit tumor angiogenesis in vitro and in vivo [13,32]. Our results indicated that DHC significantly inhibited cyclin D1 expression within 3 h of DHC treatment in a concentration- and time-dependent manner. Other cell cycle regulators appeared to be down-regulated after 12 h of DHC treatment. This study concluded that DHC-induced growth arrest of HUVECs via the down-regulation of cyclin D1, and it might play an important role in the suppression of angiogenesis.

Evidence implies that Akt signaling is essential for normal cellular G1/S phase transition and cell proliferation in endothelial cells [33,34,35,36]. Data from preclinical studies suggest that inhibitors of the PI3K/Akt signaling pathway have been reported to be potent anti-angiogenic agents and contribute to the inhibition of tumor growth [37,38,39,40,41]. Upon growth factor stimulation, Akt is activated immediately in endothelial cells. The phosphorylation of Akt at the T308 residue was mediated by PDK1, whereas phosphorylation at the S473 residue was attributed to mTORC2 [42]. The two phosphorylation sites are equally important, and lead to full activation of the Akt protein kinase. The major effectors downstream of Akt are mTORC1 and GSK-3β. The active mTORC1 induces cyclin D1 expression to promote cell growth by direct phosphorylation of the substrates, S6K1 and 4EBP1, both of which are translational regulators [29,43]. Alternatively, Akt phosphorylates and inhibits GSK-3β activity to prevent cyclin D1 degradation [7,8]. Therefore, inhibition of the Akt signaling pathway resulted in the down-regulation of cyclin D1 via a GSK-3β-degradation and mTORC1-dependent translation pathway. Most importantly, this study has identified, for the first time, the effect of DHC on the Akt/GSK-3β/cyclin D1 pathway. Additionally, DHC inhibited Akt phosphorylation at Ser473 in HUVECs, in a concentration- and time-dependent manner, suggesting that DHC is a possible competitor of mTORC2. As mentioned above, the activation of Akt is induced by various growth factors in a PI3K-dependent manner. In this study, we found that treatment with myr-Akt greatly increased the amount of Ser473 Akt present in HUVECs. Our results are similar to those for treatment with the PI3K inhibitor LY294002. We also performed the Akt kinase activity assay. However, DHC did not inhibit the ability of Akt to phosphorylate its substrate (figure S2). Therefore, DHC is a possible potent PI3K kinase or PDK1 kinase inhibitor that could display anti-angiogenic effects in vitro and in vivo.

Previous studies have shown that mTORC1 activation is not only under the regulation of Akt signaling but is also sensitive to growth factors, hypoxia, low energy, and amino acids, and it integrates these inputs to regulate cell responses [44]. However, the results showed that myr-Akt can reverse the inhibitory effects
of DHC on the mTOR pathway. This study suggests that the Akt/mTOR translational pathway is another important target responsible for DHC-induced cyclin D1 down-regulation. Previous studies have reported other targets of DHC in cancer cells including NF-κB, STAT3, and induction of ER stress [20,21,23]. In addition, activation of NF-κB has been known to regulate the expression of genes encoding angiogenic factors. Therefore, the contribution of these targets to the anti-angiogenic effect of DHC cannot be excluded. However, overexpression of myr-Akt significantly rescued the growth inhibition induced by DHC, suggesting that DHC-induced inhibition of Akt activation is responsible for the anti-proliferative effect observed in HUVECs. Thus, the mechanisms of DHC mentioned above may probably synergistically exhibit anti-angiogenic activity in vitro and in vivo.

In conclusion, this study discovered a novel mechanism by which DHC impairs angiogenesis induced by growth factors. DHC-induced cell cycle in HUVECs was arrested at the G0/G1 phase, followed by subsequent inhibition of angiogenesis in vitro and in vivo by targeting the Akt/GSK-3β and mTOR pathway. Thus, the results generated by this study suggest that DHC is a promising traditional Chinese medicine component for therapeutic intervention against angiogenesis-related diseases.

Materials and Methods

Reagents

Dehydrocostuslactone (DHC) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Propidium iodide was
Dehydrocostus lactone inhibits angiogenesis.

A. 

![Bar chart showing cell proliferation with error bars.](image)

- **Vector**
- **Myr-Akt**

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p < 0.005

B. 

![Images showing angiogenesis with annotations.](image)

- **Vector**
- **Myr-Akt**

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|        | + | + | + | + | + | + |
| **Vector** | - | - | - | + | + | + |
| **Myr-Akt** | - | - | - | + | + | + |

p < 0.05

C. 

![Bar chart showing cell proliferation with error bars.](image)

- **EGM-2**
- **NaCl (10 mM)**
- **LiCl (10 mM)**
- **DHC (3 μM)**

|        | - | - | - | - | + | + |
| **EGM-2** | - | - | - | + | + | + |
| **NaCl (10 mM)** | - | - | - | + | + | + |
| **LiCl (10 mM)** | - | - | - | + | + | + |
| **DHC (3 μM)** | - | - | - | + | + | + |

p < 0.001

p < 0.005
obtained from Sigma. Medium 199, fetal bovine serum (FBS), penicillin, streptomycin and the other tissue culture reagents were obtained from Gibco BRL Life Technologies (Grand Island, NY). Endothelial cell basal medium (EBM) and endothelial growth factors (EGM-2) were purchased from Clonetics (BioWhittaker, Walkersville, MD). The antibody against cyclin D was purchased from Calbiochem (San Diego, CA). Antibodies against CDK2, CDK4, cyclin A were purchased from Santa Cruz Biotechnology. Antibodies against p-mTOR (Ser2448), p-p70S6K (Thr421/Ser424), p-eIF4E (Ser209), p-4EBP1 (Thr37/46), p-GSK-3β

Figure 6. DHC inhibited cell proliferation and tube formation through Akt/GSK-3β pathway A, crystal violet assay. Treatment of vector group with DHC (3 μM) in EGM-2 medium significantly decreased cell proliferation numbers compared with untreated vector group. Akt overexpression significantly increased cell proliferation in DHC-treated group. B, tube formation assay. Treatment of vector group with DHC (3 μM) abrogated tube formation compared with vector control group. Akt overexpression partial reversed the inhibitory effect. C, crystal violet assay. HUVECs were treated with DHC (3 μM) and/or LiCl (10 mM). NaCl (10 mM) was as an osmolality control. Data represent from three independent experiments.
doi:10.1371/journal.pone.0031195.g006

Figure 7. Effect of DHC on mTOR signaling downstream pathway. A, Western blot analysis of phosphorylation of mTOR, p70S6K, eIF4E and 4EBP in HUVECs treated with DHC for the indicated times and concentrations. B, after transfected with the indicated plasmids, HUVECs were starved for 24 hr and then pretreated with DHC followed by 10 min of EGM-2 incubation. Phosphorylation of mTOR and 4EBP were analyzed by western blot. Data represent from three independent experiments.
doi:10.1371/journal.pone.0031195.g007
(Ser9) and Akt were purchased from Cell Signaling Technology. The antibody against p-Akt (Ser473) was purchased from Epitomics (San Francisco, CA, USA).

Cell culture

Human umbilical vein endothelial cells (HUVEC) (ScienCell Research Laboratories, San Diego, CA) were grown to confluence on 1% gelatin, and maintained in endothelial cell medium (ECM) (ScienCell Research Laboratory) supplemented with 5% FBS, 1:5 Penicillin/Streptomycin (ScienCell Research Laboratory) and 1:5 endothelial cell growth supplements (ECGs) (ECGs; Upstate Biotechnology Inc., Lake Placid, NY). Before treatment of dehydrocostuslactone in EGM-2 medium, HUVECs were starved for 24 hours in EBM-2 medium. All of the following experiments were performed in this medium.

In vivo matrigel plug assay

All animal experiments followed ethical standards, and protocols has been reviewed and approved by Animal Use and Management Committee of National Health Research Institutes (NHRI-IACUC-099003-A). In vivo angiogenesis assay was determined as blood vessel growth in the exogenous matrigel plug injected in C57BL/6 mice (6 weeks old). Briefly, matrigel (BD Bioscience) was mixed with heparin (10 units/ml), IGF-1 (40 ng/ml), EGF (40 ng/ml), bFGF (40 ng/ml) and with or without DHC and the resulting mixture was injected subcutaneously into the mice abdomens. After seven days, the animals were sacrificed and the matrigels were carefully dissected and photographed. To quantify the blood vessel formation, hemoglobin content was analyzed by Drabkin’s reagent kit (Sigma).

Crystal violet assay

The HUVECs were seeded into 96-well culture plates (5.0 × 10^3 cells per well) in triplicate. Twenty-four hours later, the culture medium was removed and replaced with fresh EGM-2 medium containing DHC at various concentrations. After 72 hours of incubation at 37°C, the cells were stained with 0.1% crystal violet/20% methanol for 10 minutes. Then the dye was eluted by 0.1 M HCl for 1 hour. Subsequently, HUVECs were harvested after treatment of DHC in EGM-2 medium for 24 hours and then replaced with EGM-2 medium with or without DHC for 10 hours. After trypsinization and fixed in ice-cold 75% methanol for a minimum of one hour at −20°C, cells were washed with PBS and resuspended in 0.2 ml DNA extraction buffer (0.2 M Na2HPO4, 0.1 M citric acid; pH 7.8) for 30 minutes. Then the cells were stained with propidium iodide solution (PI; 100 µg/ml RNase, 80 µg/ml propidium iodide, 0.1% Triton X-100) in PBS. Cell cycle distribution was determined by FACSScan flow cytometry, and data analysis was performed with CellQuest software (BD Biosciences).

Flow cytometric analysis

HUVECs were harvested after treatment of DHC in EGM-2 medium for 24 hours and then total cell lysate were prepared in a modified RIPA buffer (150 mM NaCl, 1 mM EDTA, 1% Nonidet p-40, 0.5% sodium deoxycholate, 0.1% SDS, 20 mM Tris, pH 8.0) with protease inhibitors (1 µg/ml aprotinin, 1 µg/ml leupeptin, 0.5 M NaF, 0.5 M Na3VO4, 1 mM phenylmethylsulfonyl fluoride). The cell extract proteins were separated by 8%–12% polyacrylamide gel electrophoresis followed by electroblotting onto polyvinylidene difluoride membranes. Membranes were subsequently blocked with 5% nonfat milk, washed with PBS, incubated with antibodies and detected utilizing an enhanced chemiluminescence (ECL) detection system.

Statistical analysis

The significance of differences in vivo data were analyzed by the Mann-Whitney U test; others represent the mean and SEM of at least three independent experiments. Statistical analysis was performed by the t-test, and P values less than 0.05 (* P<0.05, ** P<0.01, *** P<0.001) were considered significant.

Supporting Information

Figure S1 Effect of DHC on HUVECs migration. A, HUVECs migration after DHC treatment was assessed by wound-healing assay. Upon reaching 95% confluence, the HUVEC monolayer was scratched and cell debris was removed. Cells were cultured with EGM-2 medium and pretreated with PD98059 (10 µM) or SB203580 (10 µM) for 30 min, and then cells were treated with DHC (3 or 5 µM). After incubation for 16 h, cells were stained with crystal violet and photographed. B, Western blot analysis of the protein expression of p-p38, p-ERK1/2, p-Akt (Ser9) and Akt were purchased from Cell Signaling Technology. The antibody against p-Akt (Ser473) was purchased from Epitomics (San Francisco, CA, USA).
2, CHOP in DHC-treated HUVECs with the indicated times and concentrations. Data represent from three independent experiments.

(PDF)

Figure S2 DHC did not inhibit Akt kinase activity. Akt Kinase activity kit was purchased from Enzo Life Sciences. Data represent from three independent experiments.

(PDF)

Figure S3 Rapamycin increased the anti-proliferative effect induced by DHC. Crystal violet assay, HUVECs were treated with DHC (3 µM) and/or rapamycin (3 µM). Inhibition of mTOR activity increased the anti-proliferative effect of DHC. Data represent from three independent experiments.

(PDF)

Figure S4 Effect of DHC on early and late stage of HUVECs apoptosis was detected by flow cytometry with Annexin-V-FITC/PI dual staining. A representative histogram of flow cytometric analysis using double staining with annexin-V (FITC-A) and PI (PE-A). HUVECs were treated with DHC (5 µM) in EBM-2 basal medium for 4 hr or 24 hr. The lower right quadrants represent the cells in the early stage of apoptosis. The upper right plus left quadrants contain the cells in the late stage of apoptosis and necrosis. Data represent from three independent experiments.

(PDF)

Author Contributions

Conceived and designed the experiments: CW AT KL CT SP. Performed the experiments: CW AT CP YC SP. Analyzed the data: CW AT CT SP. Contributed reagents/materials/analysis tools: CW AT CP YC KL CT. Wrote the paper: CW AT SP.

References


