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Dietary Compound Proanthocyanidins from Chinese bayberry (*Myrica rubra* Sieb. et Zucc.) leaves inhibit angiogenesis and regulate cell cycle of cisplatin-resistant ovarian cancer cells via targeting Akt pathway

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Abstract

Ovarian cancer is the leading cause of death from gynecological malignancy and natural products have drawn great attention for cancer treatment. Chinese bayberry leaves proanthocyanidin (BLPs) with epigallocatechin-3-O-gallate (EGCG) as its terminal and major extension units is unusual in the plant kingdom. In the present study, BLPs showed strong growth inhibitory effects on cisplatin-resistant A2780/CP70 cells by inhibiting angiogenesis and inducing G1 cell cycle arrest. BLPs reduced the tube formation in HUVECs and attenuated the wound healing ability in A2780/CP70 cells. BLPs further reduced the level of ROS and targeted Akt/mTOR/p70S6K/4E-BP-1 pathway to reduce the expression of HIF-1 α and VEGF, and thus inhibited angiogenesis. Furthermore, BLPs induced G1 cell cycle arrest by reducing the expressions of c-Myc, cyclin D1 and CDK4, which was also in accordance with the flow cytometry analysis. Overall, these results indicated that BLPs could be a valuable resource of natural compounds for cancer treatment.

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Conflict of interest

The authors declare that they have no conflicts of interest.

Keywords

Proanthocyanidins; Chinese bayberry leaves; ovarian cancer; angiogenesis; cell cycle; Akt

1. Introduction

Ovarian cancer is the leading cause of death from gynecological malignancy and it is the seventh-most common cancer and the eighth-most common cause of death from cancer among women (Stewart & Wild, 2014). Surgery, chemotherapy, hormone therapy and radiation therapy are the most common treatments for ovarian cancer. Generally, two or more different types of treatments are used, which might cause severe side effects, such as fatigue, cognitive impairment and immunosuppression, to patients (Y.-I. Yang, Kim, Lee, & Choi, 2011). Furthermore, patients with later cancer stages often encounter chemoresistance and recurrence and do not respond to chemotherapies. Therefore, the 5-year survival rate for patients with ovarian cancer at later stages was less than 20% in the past 20 years (B. Li, Gao, Rankin, Rojanasakul, Cutler, Tu, et al., 2015). Other than conventional treatment, natural products have attracted great attention and have played an important role in cancer chemoprevention. Proanthocyanidins (PAs), also known as condensed tannins, are oligomers (ranging from dimer to tetramer) and polymers of flavan-3-ols and contributes to a major class of polyphenols ingested from the diet (Ou & Gu, 2014). Many studies have showed the anti-cancer properties of PAs such as inhibiting tumor angiogenesis, inducing cell cycle arrest and apoptosis, etc. (Nandakumar, Singh, & Katiyar, 2008).

Chinese bayberry (*Myrica rubra* Sieb. et Zucc.) has been cultivated in Southern China for more than 2000 years and is popular among local people. However, leaves from bayberry trees are always abandoned after harvest, which causes huge ecological waste (Y. Zhang, Chen, Wei, Gong, Li, & Ye, 2016). Chinese bayberry leaves contained rich PAs with special structures. Chinese bayberry leaves PAs (BLPs) with the mean degree of polymerization (mDP) at about 6.5 contain epigallocatechin-3-O-gallate (EGCG) as the terminal and most of their extension units, and greater than 98% of them are galloylated, which is quite unusual in the plant kingdom (Fu, Qiao, Cao, Zhou, Liu, & Ye, 2014; H. Yang, Ye, Liu, Chen, Zhang, Shen, et al., 2011; Yu Zhang, Zhou, Tao, Li, Wei, Duan, et al., 2016). Our former studies showed that BLPs exhibited strong antioxidant (Yu Zhang, Ye, Xu, Duan, Wei, Xu, et al., 2017), antiproliferative (Yu Zhang, et al., 2016) and lipid regulation capacities (Yu Zhang, Chen, Wei, Chen, & Ye, 2017). However, their functions as anti-cancer are yet to be investigated.

The ability to induce angiogenesis is considered as one of the hallmarks of cancer (Hanahan & Weinberg, 2011). The process of angiogenesis involves the migration, growth and differentiation of endothelial cells and thus causes the formation of new blood vessels from pre-existing vascular network (H. Huang, Chen, Rojanasakul, Ye, Rankin, & Chen, 2015). Angiogenesis is fundamental for tumor growth and progression because it can support cancer cells with sufficient oxygen and nutrients and allow the cancer cells to invade nearby tissues. Angiogenesis requires the binding of some signaling molecules, such as vascular endothelial growth factor (VEGF) to initiate the growth and survival of new blood vessels

(Hefler, Mustea, Könsgen, Concin, Tanner, Strick, et al., 2007). VEGF can be directly up-regulated by hypoxia-inducible factor 1 (HIF-1), which is a transcriptional factor and plays a key role in cell survival and tumor invasion. Since VEGF and HIF-1 are over-expressed in many different kinds of cancers, they are also the major targets for cancer treatment (Zhong, De Marzo, Laughner, Lim, Hilton, Zagzag, et al., 1999). Other than angiogenesis, cancer cells also exhibit defective cell-cycle checkpoints, which lead to their uncontrolled proliferation (Gabrielli, Brooks, & Pavey, 2012). Generally, the process of cell cycle goes through four phases, which are G1, S, G2 and M. Cyclin dependent kinases (CDKs), as a family of important enzymes, bind with cyclins to form the cyclin-CDK complex and thus actively regulate the progression through the cell cycle. Thus, a number of anti-cancer agents also target cell cycle regulation in cancer therapy, especially at the cyclin-CDK complex (Diaz-Moralli, Tarrado-Castellarnau, Miranda, & Cascante, 2013).

In the present study, we investigated the anti-cancer properties of BLPs by exploring their effects on angiogenesis and cell cycle in A2780/CP70 cisplatin-resistant ovarian cancer cells. The expression of VEGF, HIF-1 α and reactive oxygen species (ROS) were examined and the HUVEC tube formation assay and the wound healing assay were used to assess the anti-angiogenesis functions of BLPs. Also, major angiogenesis signaling pathways were investigated. Furthermore, how BLPs affected cell cycle was examined by flow cytometry and some key proteins involved in cell cycle phases were determined by Western blot analysis. Our data demonstrated that BLPs exhibited anti-angiogenic functions and induced G1 cell cycle arrest by targeting mainly the Akt pathway.

2. Methods and materials

2.1 Materials and reagents

Propidium iodide and 2',7'-Dichlorofluorescein diacetate were purchased from Sigma-Aldrich (Sigma, St. Louis, MO, USA). Antibodies against Akt, phospho-Akt, HIF-1 α , mTOR, phospho-mTOR, p70S6K, phospho-p70S6K, 4E-BP1, phospho-4E-BP1, CDK4, cyclin D1, c-Myc were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies against GAPDH, Erk, phospho-Erk were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). Human ovarian cancer cell line A2780/CP70 was kindly provided by Dr. Bing-Hua Jiang, Department of Microbiology, Immunology, and Cell Biology, West Virginia University, Morgantown, WV, USA. Human umbilical vein endothelial cells (HUVECs) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA).

2.2 Proanthocyanidins from Chinese bayberry leaves

BLPs were obtained according to former studies from our group (Fu, Qiao, Cao, Zhou, Liu, & Ye, 2014; H. Yang, et al., 2011; Yu Zhang, et al., 2016). The total phenolic content of BLPs is 378.28 ± 0.97 milligrams of gallic acid equivalents per gram dry weight. The mDP of BLPs is 7.3 ± 0.1 . BLPs contain 5.4% monomeric, 10.9% dimeric, 13% trimeric, 47.8% tetrameric PAs and 22.9% polymeric PAs or other phenolics.

2.3 Cell culture

Human ovarian cancer cell line A2780/CP70 and human normal ovarian cells IOSE-364 were cultured in RPMI 1640 medium (Sigma, St. Louis, MO, USA) supplemented with 10% US-qualified fetal bovine serum (Invitrogen, Grand Island, NY, USA) at 37 °C with 5% CO₂. HUVECs were cultured in vascular basal medium supplemented with endothelial cell growth kit-VEGF (ATCC, Manassas, VA, USA) at 37 °C with 5% CO₂.

2.4 Cell viability assay

A2780/CP70 and IOSE-364 cells were seeded into 96-well plates at a density of 2×10^4 per well in medium with 10% FBS at 37 °C with 5% CO₂ and were attached to the bottom overnight and then treated with BLPs at different concentrations for 24 h. Cell viability was determined by using CellTiter 96 Aqueous One Solution Cell Proliferation assay (Promega, Madison, WI, USA) based on the manufacturer's instructions. The results were expressed as a percentage compared to control cells (vehicle treatment).

2.5 Flow cytometry analysis for cell cycle

Cells were seeded at the density of 8×10^5 per well in the medium with 10% FBS at 37 °C with 5% CO₂ in the 60-mm plates and were attached to the bottom overnight. Afterwards, cells were starved for 24 h and treated with BLPs at different concentrations for another 24 h. Then, cells were digested by trypsin and collected by centrifugation and washed with cold PBS. The cell pellets were suspended in 70% ethanol at -20 °C overnight. Afterwards, cells were washed with PBS and incubated with 180 µg/mL RNase A at 37 °C for 20 min and stained with 50 µg/mL propidium iodide (final concentration) for 15 min. Flow cytometry (FACSCaliber system, BD Biosciences) was used for detection. Data were analyzed by using FCS Software (De Novo Software, Los Angeles, CA).

2.6 Enzyme linked immunosorbent assay for VEGF

A2780/CP70 cells were seeded into 96-well plates at a density of 2×10^4 per well in medium with 10% FBS at 37 °C with 5% CO₂ and were attached to the bottom overnight and then treated with BLPs at different concentrations for 24 h. Afterwards, cell culture supernatants were collected. The concentration of VEGF was determined by a human VEGF Duo-set ELISA kit (R&D Systems, Minneapolis, MN, USA) based on the instructions of manufacturer, normalized to total protein levels, and expressed as a percentage of the untreated control.

2.7 Western blot assay

Equal amounts of proteins were separated by SDS-polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride filters membrane (GE Healthcare, Chicago, IL, USA). Membranes were blocked with 5% of nonfat milk with TBST for 1 h and then were incubated with the primary antibody over-night at 4 °C. Afterwards, membranes were washed three times with TBST and then incubated with the secondary antibody for 1 h. Bands were detected by the ECL Western blot detection reagents (Thermo Fisher Scientific, Waltham, MA, USA) and exposed to a Mini-Protean 3 System (Bio-Rad, Atlanta, GA, USA).

2.8 HUVEC tube formation assay

The HUVEC tube formation assay was performed based on previous studies with slight modification (Gao, Rankin, Tu, & Chen, 2016; H. Huang, Chen, Rojanasakul, Ye, Rankin, & Chen, 2015). A2780/CP70 cells were seeded into 96-well plates at a density of 2×10^4 /well and incubated overnight. Afterwards, cells were treated with BLPs at different concentrations for 24 h and then the conditioned medium was collected. Growth factor-reduced Matrigel (BD Biosciences, San Jose, CA, USA) was added into 96-well plates and incubated at 37 °C with 5% CO₂ for 30 min to allow gel formation. HUVECs were seeded at the concentration of 1.5×10^4 per well (90 µL vascular cell basal medium + 10 µL collected conditioned medium) into Matrigel beds. After a 6-h incubation at 37 °C with 5% CO₂, each well was photographed. The tube length was measured by the NIH ImageJ software (NIH, Bethesda, MD, USA) and was normalized to that of the control.

2.9 Intracellular ROS staining and measurement

Intracellular ROS levels in both BLPs-treated and control cells were measured via the DCFH-DA assay based on some previous studies with slight modification (B. Li, et al., 2015). The sub-confluent A2780/CP70 cells were treated with BLPs (2.5, 5, or 10 µg/mL) for 24 h, and then cells were incubated with 25 µM DCFH-DA for 30 min at 37 °C with 5% CO₂. Afterwards, cells were washed with PBS (pH 7.4) twice and the images were visualized and photographed under a fluorescent microscope (ZEISS, NY, USA). The fluorescence intensity was measured at 538 nm (emission wavelength) and 485 nm (excitation wavelength) by a SynergyTM HT Multi-Mode Microplate Reader (BioTek). ROS generation was normalized by the total protein level, and was expressed as percentage of the untreated control.

2.10 Wound healing assay

The wound healing assay was performed based on a previous study with slight modification (Tsai, Hsieh, Lee, Hsu, Wang, Kuo, et al., 2015). Cell were seeded in 12-well plate to 70–80% confluence. A pipet tip was used to scratch the cell monolayer straightly. The cells were afterwards washed twice with PBS to get rid of debris. Then BLPs at different concentrations dissolved in the medium without serum were added to the cells. The control contained only the medium without serum or any other treatment. All of the cells were photographed at 0 and 24 h with a fluorescence microscope (ZEISS).

2.11 Statistical analysis

Results are presented as the mean $\pm$ standard deviation (SD) for at least three replicates for each sample. Statistical analyses were performed using the SPSS program, version 17.0 (SPSS Inc., 2009). Data were analysed by ANOVA and significant differences were set at $p < 0.05$.

3. Results

3.1 Effects of BLPs on cell growth of A2780/CP70 and IOSE-364 cells

To investigate the cytotoxic effects of BLPs on human ovarian cancer cells A2780/CP70 and normal ovarian cells IOSE-364, the CellTiter 96 Aqueous One Solution Cell Proliferation assay was performed after treating both A2780/CP70 and IOSE-364 cells with BLPs at different concentrations. As shown in Fig. 1, BLPs could inhibit the proliferation of both A2780/CP70 and IOSE-364 cells in a dose-dependent manner ($p < 0.01$). The cell viability with BLPs treatment (4–10 $\mu\text{g/mL}$) for 24 h varied from $91.76 \pm 5.79\%$ to $49.25 \pm 2.21\%$ for A2780/CP70 cells (Fig. 1A), while, from $92.29 \pm 1.50\%$ to $80.57 \pm 1.74\%$ for IOSE-364 cells (Fig. 1B). These results suggested that BLPs induced less cytotoxic effects on IOSE-364 cells than those on A2780/CP70 cells.

3.2 BLPs inhibited angiogenesis via reducing the production of VEGF, HIF-1 α and ROS in A2780/CP70 cells

VEGF as a growth factor plays an important role in tumor vascular development and maintenance, and it was investigated based on the ELISA assay. Fig. 2D shows that the VEGF secretion was significantly decreased in a dose dependent relationship after the BLPs treatment ($p < 0.05$). BLPs at the concentration of 2.5, 5 and 10 $\mu\text{g/mL}$ reduced about 33.32%, 63.25% and 80.30% of VEGF secretion compared with the control, respectively. VEGF can be directly regulated by HIF-1 α , which was also detected by the western blot assay in the present study. Fig. 2A shows that BLPs dose-dependently inhibited the expression of HIF-1 α and the inhibition rate reached 73.04% after treatment with BLPs at 10 $\mu\text{g/mL}$. Furthermore, VEGF production is also associated with ROS production within cells. It has been reported that ROS generated from mitochondria are essential for stabilization of HIF-1 α and induction of VEGF (S. Huang, Yang, Liu, Gao, Huang, Hu, et al., 2012). Fig. 2B shows that BLPs significantly reduced intracellular ROS generation by showing weaker fluorescence intensity as its concentration increased. These results indicated that BLPs might inhibit tumor angiogenesis by targeting VEGF.

3.3 BLPs inhibited HUVEC tube formation and attenuated the wound healing ability of A2780/CP70 cells

The anti-angiogenesis property of BLPs was further investigated by the HUVEC tube formation assay, which is widely used as an *in vitro* model to study angiogenesis (Arnaoutova & Kleinman, 2010). As it is shown in Fig. 3A, HUVECs in the control group obviously exhibited connected tube-like networks as a result of angiogenesis. However, treatment with BLPs dose-dependently inhibited the HUVEC tubes formation by showing significantly shorter tube length and fewer networks (Fig. 3A). Since angiogenesis not only is vital in tumor growth and development, but also plays an important role in wound healing (Hanahan & Weinberg, 2011). Therefore, the effect of BLPs on the wound healing ability of A2780/CP70 cells was further investigated. Fig. 3B shows that cells in the control group obviously migrated after 24 h as the gap between the scratch was much narrower than that at 0 h. However, the gaps between the scratch of BLPs at 5 and 10 $\mu\text{g/mL}$ at 24 h did not show obvious differences compared with those at 0 h, which indicated that BLPs inhibited the wound healing process and reduced cell migration in ovarian cancer A2780/CP70 cells.

3.4 Regulation of proteins of angiogenic pathways by BLPs in A2780/CP70 cells

The PI3K/Akt/mTOR signaling pathway has been reported to play an important role in tumor angiogenesis. Activation of the PI3K/Akt/mTOR signaling pathway leads to the elevated expression of HIF-1 and VEGF and thus stimulates vasculogenesis and angiogenesis (Gao, Rankin, Tu, & Chen, 2016). Also, Erk as one of the key targets for therapeutic intervention for cancer showed an essential role in tumor angiogenesis as many studies reported (Saxena, Sharma, Ding, Lin, Marra, Merlin, et al., 2007). Therefore, key proteins in both PI3K/Akt/mTOR and Erk pathways were investigated in the present study (Fig. 4). After treatment with BLPs (2.5, 5 and 10 µg/mL) for 24 h, the expression of phosphorylated Akt was significantly reduced in a dose dependent manner ($p < 0.05$). Compared to the control, BLPs at 2.5, 5 and 10 µg/mL reduced the phosphorylation of Akt at about 28.45, 68.03 and 93.43%, respectively and thereby significantly inactivated Akt. Afterwards, BLPs treatment interfered with the downstream proteins of Akt signaling pathway by significantly reduced the expression of p-mTOR, p-p70S6K, p-4E-BP-1 and therefore inactivated their activities. Besides, the expression of p-Erk was also suppressed by BLPs, however, at a weaker extent compared with that of p-Akt. These results suggested that BLPs mainly suppressed the Akt signaling pathways and thereby downregulated the expression of HIF-1 α to inhibit process of angiogenesis.

3.5 BLPs induced G1 phase cell cycle arrest in A2780/CP70 cells

Since the cell cycle of cancer cells is faster than that of normal cells, therefore, cell cycle arrest influences cancer cells more than normal cells (B. Li, et al., 2015). The effects of BLPs on cell cycle phase distribution was further analyzed by flow cytometry with propidium iodide staining. As it is shown in Table 1, treatment with BLPs obviously increased G1 phase distribution and decreased G2 phase distribution, however, exhibited no obvious effects on S phase distribution ($p < 0.05$). The percentage of G1 phase increased about 42.72% after treatment with BLPs at 10 µg/mL compared with that of the control. Similar results were also observed in a previous study that EGCG as a potent monomer induced G1 arrest in different cancer cell models (Du, Zhang, Wen, Yu, Calway, Yuan, et al., 2012). These results indicated that G1 cell cycle arrest might also be involved in the interference of BLPs in the growth and viability of A2780/CP70 cells.

3.6 Regulation of cell cycle G1-related proteins by BLPs in A2780/CP70 cells

Based on the analysis of flow cytometry that BLPs induced a G1 arrest in A2780/CP70 cells, we further evaluated how BLPs affected the key proteins in the G1 cell cycle progression such as cyclin D1, CDK4, glycogen synthase kinase 3 β (GSK-3 β), forkhead box (FOXO) proteins, c-Myc and Akt by Western blot analysis. Except for regulating angiogenesis, Akt has been reported to play an key role in G1 cell cycle by regulating several target proteins, such as GSK-3 β , FOXO proteins and c-Myc to regulate the expression of cyclin D1 and CDK4 (Luo, Manning, & Cantley, 2003). As it is shown in Fig. 5, treatment with BLPs dose-dependently reduced the expression of p-Akt and c-Myc, which further lead to the downregulation of cyclin D1 and CDK4 ($p < 0.05$). Particularly, BLPs at 10 µg/mL inhibited about 57.7% and 58.1% of the expression of cyclin D1 and CDK4 compared with that of the control, respectively. However, BLPs showed no obvious effects on the expression of

phospho-GSK-3 β and phospho-FoxO1/Fox3. Taken together, BLPs possibly targeted Akt and c-Myc to inhibit the expression of cyclin D1 and CDK4 and thus induced G1 cell cycle arrest.

3. Discussion

Ovarian cancer as one of the most common cancers occurring in women causes more deaths than any other cancer of female reproductive system. Although chemotherapy could be helpful in curing ovarian cancer, it might lead to resistance to anticancer agents and also induce some adverse effects (Y.-I. Yang, Kim, Lee, & Choi, 2011). This calls for the demand of new strategies for ovarian cancer treatment. The importance of natural products is emerging in combating cancers nowadays with extensive studies showing that various types of polyphenols interfered tumor proliferation and metastasis (Y. Li, Wicha, Schwartz, & Sun, 2011). BLPs were reported to exhibit potent antioxidant, anti-proliferative and anti-hyperlipidemic properties based on some former studies (Yu Zhang, Chen, Wei, Chen, & Ye, 2017; Yu Zhang, et al., 2016), however, its function in anti-angiogenesis and cell cycle arrest in ovarian cancer cells has yet to be investigated. Therefore, in the present work, we demonstrated that BLPs could strongly inhibited the growth of cisplatin-resistant A2780/CP70 ovarian cancer cells, and such effects might be due to the BLPs-induced anti-angiogenesis and G1 cell cycle arrest.

Angiogenesis, also known as the formation of new blood vessels from pre-existing blood vessels plays an important role in tumorigenesis and is proposed as one of the six hallmarks of cancer (Hanahan & Weinberg, 2011). Cancer cells are usually under greater hypoxia and oxidative stress than normal cells and they can produce excessive ROS by altering multiple metabolic pathways (Szatrowski & Nathan, 1991). The synergistic effects of hypoxia and ROS lead to the augmented expression of VEGF, which is one of the pro-angiogenic factors and plays an important role in angiogenesis (Hoeben, Landuyt, Highley, Wildiers, Van Oosterom, & De Bruijn, 2004). Thus, anti-VEGF therapies is one of the target for cancer treatments, including ovarian cancer. Based on the result of the ELISA assay, BLPs dose-dependently inhibited VEGF secretion with the inhibition rate at about 80.3% compared with the control at 10 μ g/mL (Fig. 2C). The BLPs-induced inhibition of VEGF might be due to the inhibition effects of BLPs on ROS. A large amount of ROS produced from tumor cells can trigger angiogenesis by elevating VEGF and matrix metalloproteinase activities (Arbiser, Petros, Klafter, Govindajaran, McLaughlin, Brown, et al., 2002). BLPs significantly reduced the ROS production from A2780/CP70 cells in a dose dependent manner, which was also in accordance with the VEGF assay (Fig. 2B & 2C).

However, the BLPs-induced inhibition of VEGF might be more directly due to the effects of BLPs on HIF-1 α , which is a subunit of heterodimeric transcription factor hypoxia-inducible factor 1. HIF-1 α is the master transcriptional regulator of cellular growth and development in response to hypoxia. It has been reported that HIF-1 α can directly target VEGF and up-regulation of HIF-1 α promotes VEGF expression and thus induce tumor angiogenesis (H. Huang, Chen, Rojanasakul, Ye, Rankin, & Chen, 2015). In the present study, BLPs inhibited the expression of HIF-1 α in a dose-dependent relationship (Fig. 2A). Such result was consistent with many previous studies, which revealed that PAs, particularly EGCG inhibited

angiogenesis by reducing the expression of HIF-1 α (Mojzis, Varinska, Mojzisova, Kostova, & Mirossay, 2008). EGCG was reported to inhibit the prolyl hydroxylation of HIF-1 α and thus inhibit its activity (Thomas & Kim, 2005). Also, EGCG contains the hydroxylation of its A ring at positions 5 and 7 and of its B ring at the 4' position, which were reported to be important for the inhibition of hypoxia-induced VEGF expression (Ansó, Zuazo, Irigoyen, Urdaci, Rouzaut, & Martínez-Irujo, 2010). Thus, the strong inhibitory effects of BLPs on HIF-1 α and VEGF might be associated with the special structure of BLPs, which contain EGCG as its major units.

By inhibiting the activities of HIF-1 α and VEGF, the HUVEC tube formation assay and wound healing assay further exhibited that BLPs was able to attenuate the process of angiogenesis. Treatment with BLPs reduced the HUVEC tube formation in a dose-dependent manner (Fig. 3A). After treatment with BLPs at 10 μ g/mL, the tube length only reached $37.8 \pm 2.7\%$ of that of the control. Besides, angiogenesis is also involved in the process of wound healing (Erba, Ogawa, Ackermann, Adini, Miele, Dastouri, et al., 2011). Treatment with BLPs attenuated the wound healing ability of A2780/CP70 cells by showing a much wider gap between the two sides of cells after being scratched (Fig. 3B). Taken together, results from the ELISA assay (for the detection of VEGF), ROS staining assay, western blot assay (for the detection of HIF-1 α), HUVEC tube formation assay and wound healing assay were in consistency and suggested that BLPs has a strong potency to inhibit angiogenesis in A2780/CP70 ovarian cancer cells.

In order to figure out how BLPs exhibited the anti-angiogenesis effects on A2780/CP70 cells, key proteins of the angiogenesis signaling pathways were investigated. There are multiple pathways targeting HIF-1 α /VEGF to regulate angiogenesis. Akt has been reported to participate in the process of angiogenesis and tumor development by activating HIF-1 α and VEGF both *in vitro* and *in vivo* (J. Chen, Somanath, Razorenova, Chen, Hay, Bornstein, et al., 2005). Activation of Akt subsequently phosphorylates mTOR and thus activates it, which then leads to the activation of two downstream proteins, p70S6K and 4E-BP1. mTOR as an ATP and amino acid sensor to balance nutrient availability and cell growth plays an important role in tumor development as well. It is under investigation as a potential target for cancer treatment. p70S6K known as a mitogen activated Ser/Thr protein kinase and 4E-BP1 known as the translation repressor protein are both vital for cell growth and can be regulated by mTOR (Gao, Rankin, Tu, & Chen, 2016). In the present study, we proved that BLPs could significantly reduce the phosphorylation of Akt in a dose-dependent manner and afterwards decreased the phosphorylation of mTOR, p70S6K and 4E-BP1. As a result, the expression of HIF-1 α and VEGF were strongly inhibited by BLPs. These results indicated that Akt/mTOR/p70S6K/4E-BP1 might be a potential pathway contributing to the anti-angiogenic effects of BLPs, which were also in accordance with some previous studies that Akt pathway involved in the anti-angiogenic function of some natural products, such as EGCG, cranberry PAs, quercetin and kaempferol, etc. (Cerezo-Guisado, Zur, Lorenzo, Risco, Martín-Serrano, Alvarez-Barrientos, et al., 2015; A. Y. Chen & Chen, 2013; Kim, Singh, Singh, Demartino, Brard, Vorsa, et al., 2012)

Abnormal cell cycle control contributes to the uncontrolled proliferation and growth of tumor, and it is also regarded as a representative character of cancer (Gabrielli, Brooks, &

Pavey, 2012). During the first gap phase (G1) of the cell division cycle, mitogenic stimulation initiates DNA synthesis (S phase), and afterwards cells are committed to complete the cycle and divide. In the early G1 phase, D type cyclins (D1, D2 and D3) assemble with CDK 4 and 6 into holoenzyme complexes that regulate the ordered progression through the cell cycle (Malumbres & Barbacid, 2009). Thus, many investigations focused on G1 cell cycle checkpoint by targeting the cyclin D1-CDK4 complex as one of the anti-cancer therapeutics. Results by flow cytometry revealed that BLPs dose-dependently induced G1 arrest by showing increasing G1 cell cycle phase distribution compared with the control ($p < 0.05$) (Table 1). The BLPs-induced G1 arrest might be associated with the downregulation of cyclin D1-CDK4 complex. Fig. 5 shows that BLPs treatment significantly reduced the expression of cyclin D1 and CDK4 ($p < 0.05$). Akt pathway not only plays a key role in regulating tumor angiogenesis, but also contributes to the regulation of cell cycle progression, particularly at the G1/S transition by targeting Cyclin D1-CDK4 complex (Shimura, Noma, Oikawa, Ochiai, Kakuda, Kuwahara, et al., 2012). Activation of Akt promotes the expression of c-Myc and inhibits GSK3 and FOXO, and thus stabilizes cyclin D1 and accelerates G1 progression (Luo, Manning, & Cantley, 2003). In the present study, BLPs treatment reduced the expression of phosphorylated-Akt and c-Myc in a dose dependent relationship, however, did not show obvious effects on the expression of phospho-GSK-3 β and phospho-FoxO1/Fox3. Some previous studies also revealed that EGCG, green tea extracts and grape seed PAs extracts induced G1 cell cycle arrest by reducing the expression of cyclin D1-CDK4 complex (Di Domenico, Foppoli, Coccia, & Perluigi, 2012). Thus, these results were in consistency with previous studies and indicated that the BLPs-induced G1 cell cycle arrest might be related with the inactivation of Akt and c-Myc, which lead to the reduction of cyclin D1-CDK4 complex.

4. Conclusion

This research demonstrated that BLPs had strong inhibitory effects on the growth of cisplatin-resistant ovarian cancer cells by exhibiting anti-angiogenic function and induction of G1 cell cycle arrest via the Akt pathway. BLPs exerted the anti-angiogenic function by reducing the generation of ROS and attenuating the Akt/mTOR/p70S6K/4E-BP1 pathway and therefore reduced the expression of HIF-1 α and VEGF. Furthermore, BLPs also targeted Akt/c-Myc/cyclin D1-CDK4 to induce G1 cell cycle arrest in A2780/CP70 cells. Based on these results, BLPs could be a potential natural compound for the treatment for ovarian cancer patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Effects of BLPs on cell growth in human ovarian cancer cells A2780/CP70 and normal ovarian cells IOSE-364. **(A)** BLPs inhibited the viability of A2780/CP70 cells in a dose dependent manner. Different letters refer to statistically significant differences at $p < 0.01$. **(B)** BLPs inhibited the viability of IOSE-364 cells in a dose dependent manner. Different letters refer to statistically significant differences at $p < 0.01$.

Fig. 2.

(A) BLPs reduced the expression of HIF-1α in A2780/CP70 cells. Cells were treated with BLPs at different concentrations for 24 h. The expression of HIF-1α was detected by Western blot analysis and quantified by Image J software. Results were from three independent experiments and were expressed as means ± SD. Different letters refer to statistically significant differences at $p < 0.05$. (B) BLPs inhibited ROS production in A2780/CP70 cells. A2780/CP70 cells were treated with BLPs for 24 h and then incubated with DCFH-DA (25 μM) for 30 min. The fluorescence intensity was measured by a fluorescence reader. Results were from three independent experiments and were expressed as means ± SD. Different letters refer to statistically significant differences at $p < 0.05$. (C) BLPs inhibited VEGF secretion in A2780/CP70 cells in a dose dependent manner. Different letters refer to statistically significant differences at $p < 0.05$.

Fig. 3.

(A) A2780/CP70 cells-induced HUVEC tube formation was inhibited by BLPs. A2780/CP70 cells were seeded and treated with BLPs at different concentrations for 24 h and then the medium was collected. HUVEC cells were harvested and seeded onto the gelled Matrigel beds. The collected conditioned cell culture medium was added to the HUVEC cells and incubated for 6 hours and were photographed. The tube length was measured by the NIH ImageJ software and was normalized to that of the control. Results were from three independent experiments and were expressed as means $\pm$ SD. Different letters refer to statistically significant differences at $p < 0.05$. (B) BLPs regulated wound healing ability of A2780/CP70 cells. Cell were seeded in 12-well plate and a pipet tip was used to scratch the cell monolayer straightly. BLPs at different concentrations dissolving in the serum-free medium were added to the cells. The control contained only the serum-free medium. All of the cells were photographed at 0 and 24 h, respectively.

Fig. 4.

BLPs regulated angiogenesis-related proteins in A2780/CP70 cells. Cells were treated with BLPs at different concentrations for 24 h. p-mTOR, mTOR, p-p70S6K, p70S6K, p-Akt, Akt, p-Erk, Erk, p-4E-BP-1, 4E-BP-1 and GAPDH protein expressions were detected by Western blot analysis and quantified by Image J software. Results are representative of three independent experiments and are expressed as mean $\pm$ SD. Relative activation of 4E-BP-1, Erk, Akt, p70S6K, mTOR was expressed as dividing their phosphorylated amount by that of their corresponding total expressions. Different letters refer to statistically significant differences at $p < 0.05$.

Fig. 5.

BLPs regulated G1-related proteins in A2780/CP70 cells. p-Akt, Akt, c-Myc, cyclin D1, CDK4 and GAPDH protein expressions were detected by Western blot analysis and quantified by Image J software. Results are representative of three independent experiments and are expressed as mean $\pm$ SD. Relative activation Akt was expressed as dividing their phosphorylated amount by that of their corresponding total expressions. Different letters refer to statistically significant differences at $p < 0.05$.

Fig. 6. Proposed mechanism of BLPs-induced anti-angiogenesis and cell cycle regulation via the Akt pathway.

Table 1

Cell cycle phase distribution of A2780/CP70 cells with BLPs treatment.

BLPs (µg/mL)	Cell cycle phase distribution		
	%G1	%G2	%S
0	35.32 ± 0.54 ^d	22.86 ± 0.26 ^a	41.82 ± 0.28 ^a
2.5	37.75 ± 2.10 ^c	24.85 ± 0.90 ^a	37.40 ± 1.28 ^b
5	42.10 ± 0.80 ^b	18.10 ± 0.95 ^b	39.80 ± 1.02 ^{ab}
10	50.41 ± 1.10 ^a	13.02 ± 1.30 ^c	36.57 ± 1.10 ^c

* Data are expressed as mean values ± SD (n = 3). Different letters in each column refer to statistically significant differences at p < 0.05.