Ampelopsin Inhibits Breast Cancer Cell Growth through Mitochondrial Apoptosis Pathway

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Ampelopsin, a flavonoid with a wide variety of biological activities, has been proposed to be a potent antitumor agent. However, the mechanism by which Ampelopsin shows anti-breast cancer activity remains unclear. Therefore, this study will explore the mechanism of Ampelopsin’s anti-breast cancer activity by culturing MDA-MB-231 and MCF-7 breast cancer cells. Cell Counting Kit-8 (CCK-8) method and plate cloning method were used to detect the proliferation inhibition of breast cancer cells. Fluorescence microscopy was used to detect mitochondrial membrane potential (MMP). 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) method was used to determine the content of intracellular reactive oxygen species (ROS). Hoechst 33258 staining was used to detect the apoptotic morphological changes. Transmission electron microscope was used to observe the mitochondrial structure. Western blot was used to detect the protein expression of Bax and Bcl-2. The results showed that Ampelopsin could significantly inhibit the proliferation of breast cancer cells, and promote cells apoptosis. In addition, the occurrence of apoptosis in breast cancer cells was associated with mitochondrial dysfunction, including the loss of mitochondrial membrane potential, the production of large amounts of reactive oxygen species, and the up-regulation of Bax/Bcl-2 expression. In conclusion, Ampelopsin-induced mitochondria damage leads to loss of mitochondria membrane potential, overproduction of ROS and activation of Bax, increasing mitochondria membrane permeability and ultimately inducing breast cell apoptosis. These findings provided a new perspective on the role of Ampelopsin in breast cancer prevention and treatment.

Key words Ampelopsin; breast cancer; proliferation; mitochondrial apoptosis

INTRODUCTION

Breast cancer is the most common cancer and the leading cause of cancer death in women, accounting for approx. 2088849 new cases and 626679 deaths.1) The main clinical treatments for breast cancer include radiotherapy, surgical resection, chemotherapy and molecular targeted therapy.2) Currently, improvements in early diagnosis and treatment strategies have significantly improved the prognosis for patients. However, a considerable number of patients will eventually develop tumor recurrence and drug resistance.3,4) Thus, it is important to develop new and alternative therapeutic drugs for the treatment of breast cancer.

MCF-7 and MDA-MB-231 are human breast cancer cell lines with different metastatic potential. MCF-7 is an in-situ estrogen receptor (ER)-positive breast cancer cell line, with a lower degree of malignancy than MDA-MB-231, so it is less likely to metastasize. However, MDA-MB-231 is a typical cell line of triple negative breast cancer (TNBC), which is negative for ER, progesterone receptor (PR) and epidermal growth factor receptor 2 (HER2).4) TNBC does not respond to endocrine therapies or other targeting agents. To explore more chemicals with antitumor effects, especially for TNBC, is of great clinical value. A study reported that DS-8201A had anti-tumor effect on cancer expressing HER2.5)

Flavonoids are a group of naturally occurring polyphenol compounds found in fruits, vegetables and beverages derived from plants. Due to flavonoids have selective cytotoxicity to cancer cells and low toxicity to normal cells, there is considerable interest in developing it as potential cancer therapeutics.6) Ampelopsin, also known as dihydromyricetin (DHM), is a flavonoid with high content in the medicinal and edible plant rattan tea (also known as mildew tea). It was found that Ampelopsin (up to 20% (w/w)) is the most abundant flavonoid in the tender stems and leaves (measured by dry weight).7) Ampelopsin has a variety of biological activities, such as anti-inflammatory, antimicrobial, anti-cancer, hepatoprotective and lipid metabolism regulation with high safety. In animal model, even at a 300mg/kg body weight (b.w.) dose, Ampelopsin also showed low toxicity.8,9) However, the exact anti-tumor mechanism of Ampelopsin against breast cancer remains to be elucidated.

Inducing apoptosis of cancer cells is one of the most prominent properties of chemotherapeutics. In addition to the extrinsic pathway mediated by death receptors, mitochondrial-induced intrinsic apoptosis has been studied for many years. Mitochondria are double membrane organelles that produce most of the cellular energy in the form of ATP.10) At same time, mitochondria play an active role in apoptosis through releasing various mitochondrial proteins known as apoptogens.

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This process is closely associated with Bcl-2 family members, changes in mitochondrial membrane potential and imbalance of mitochondria dynamics.11-13

In this study, we aimed to explore the inhibitory effects and possible molecular mechanisms of Ampelopsin on the growth of MDA-MB-231 and MCF-7 cells. Our results indicated that Ampelopsin inhibited the proliferation in two types of breast cancer cells. Ampelopsin treatment may induce mitochondrial apoptosis through regulation of the expression of Bax and Bcl-2. This may result from the loss of mitochondrial membrane potential, accompanied with inhibition of ATP production and increased mitochondrial fission.

MATERIALS AND METHODS

Reagents and Antibodies Ampelopsin (HPLC ≥98%) was purchased from Chengdu Manster Company (Chengdu, China). Ampelopsin was dissolved in dimethyl sulfoxide (DMSO) as a stock solution. The final concentration of DMSO was kept below 0.1% in all cell cultures. Cell Counting Kit-8 (CCK-8) was a product of Dojindo Laboratories (Kumamoto, Japan). The antibodies used in this study were as follows: Primary antibody against Bax was obtained from Cell Signaling Technology, Inc. (U.S.A.). Antibodies against Bcl-2 and Fis 1 were purchased from Proteintech Group, Inc. (U.S.A.). Antibody against β-actin was a product from Beyotime Institute of Biotechnology (Haimen, Jiangsu, China).

Cell Lines and Maintenance of Cell Culture MCF-7 breast cancer cells were generously provided by Ms. Jun Tang (The First Affiliated Hospital of Chongqing Medical University). Prof. Xuemei Lian (School of Public Health and Management, Chongqing Medical University) provided the MDA-MB-231 breast cancer cells. Breast cancer cells were maintained at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. Cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), which were purchased from Gibco (Thermo Fisher, Inc., MA, U.S.A.).

Cell Proliferation Assay Cells (1 × 10⁴ cells per well) were seeded into 96-well plates and allowed to attach, and then treated separately with various concentrations of Ampelopsin (0, 20, 40, 60, 80, 100 μM) for 24 h in a 5% CO₂ incubator at 37°C. Cell proliferation was assessed using CCK-8 (10 μL per well) assay according to the manufacturer’s instructions. Then the optical density (OD) value of each well was detected at the absorption wavelength of 450 nm in the microplate analyzer. Cell viability = (Treated Group OD – Blank Group OD)/(Control Group OD – Blank Group OD) × 100%.

Colony Formation Assay Cells were inoculated in 6-well plates at an initial density of 1 × 10⁴ in 2-mL medium for 16-18h. Cells were treated with or without various concentrations of Ampelopsin for 24h. Then the medium was removed and the cells grew in the complete medium for 7–10d until different colonies were formed. Colonies were washed with phosphate buffered saline (PBS), fixed with 4% paraformaldehyde 20min and then stained with 2.5% crystal violet for 15min. After PBS washing and air drying, the colonies were imaged and recorded.

Hoechst 33258 Staining Hoechst 33258 staining was used to observe morphological changes of the nucleus caused by apoptosis. Cells (1 × 10⁶ cells per well) were seeded in 6-well plate and allowed to attach overnight. Subsequently, the cells were treated with or without various concentrations of Ampelopsin for 24h, fixed with 4% paraformaldehyde for 20min, and then stained with Hoechst 33258 for 25min. The staining solution was discarded and the cells were rinsed with PBS for at least 3 times. Photographs were taken and recorded under an inverted fluorescence microscope.

Mitochondrial Membrane Potential (MMP) Assay MMP was determined by staining with JC-1 fluorescent probe (Beytome Institute of Biotechnology). Briefly, cells were treated as previously described in 2.5, and then discarded the culture medium, added 1 mL of cell culture medium and 1 mL of JC-1 (1 : 1000 dilution), mixed thoroughly, and incubated in an incubator for 20min. After ice-cold PBS washing, cells were observed with inverted fluorescence microscope and fluorescent images were captured. The green/red fluorescence ratio was quantitatively analyzed by NIH Image J software.

Intracellular ROS Production Assay We used the oxidant-sensing fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) (Beyotime Institute of Biotechnology) to measure intracellular ROS generation. Briefly, cells were treated as described in 2.5, and then incubated with DCFH-DA (1 : 1000 dilution) for 30min at 37°C. After that, it was washed with RPMI 1640 medium for 3 times and the images were captured by inverted fluorescence microscope. As mentioned above, the NIH Image J software was used for quantitative analysis.

Observation of Morphological Changes of Mitochondria Approximately 1 × 10⁶ cells/mL were treated with 40 μM Ampelopsin or vehicle, then collected MDA-MB-231 cells washed twice with PBS, and fixed overnight in fresh 2.5% glutaraldehyde at 4°C. The samples were fixed with 2% osmium tetroxide in NaCac, stained with 2% uranyl acetate, dehydrated with graded ethanol series, and embedded with Epon-Araldite resin. The slices were obtained using Leica EM UC6 Micro-systems and stained with uranyl acetate and lead citrate. The ultrastructure of the cells was observed with Philips CM-120 transmission electron microscope (TEM).

Cellular ATP Level Assay ATP levels were detected by the ATP Detection Kit (Beyotime Institute of Biotechnology). With known ATP concentrations (0.01, 0.03, 0.1, 0.3, 1.0, 3.0, 10μM), a standard curve was plotted, which was used to determine the ATP content of the cells. Briefly, after treatment of various concentrations of Ampelopsin for 24h, cells were collected and washed with PBS. After cell lysis, the cells were centrifuged at 12000 × g and 4°C for 5min. The supernatant was collected and 100 μL ATP detection solution was added. The chemiluminescence values were determined by fluoroscope (Thermo Fisher Scientific, Inc.). ATP levels were expressed as a percentage of the control, which was set at 100%.

Western Blotting Cells (1 × 10⁶ cells/mL) were treated with or without Ampelopsin for 24h. Afterwards, they were harvested and lysed in cell lysis buffer (Beyotime Institute of Biotechnology) containing the protease inhibitor phenylmethylsulfonyl fluoride (PMSF). Total protein was quantified by using bicinchoninic acid (BCA) assay according to the instruction of protein assay kit (Beyotime Institute of Biotechnology). The protein lysate samples (20μg) were isolated with 10–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a polyvinylidene fluoride membrane. The membrane was incubated with 5% skimmed milk at room temperature for 2h to block the
nonspecific binding sites, and then incubated with respective primary antibodies overnight at 4 °C. The following antibodies were used: Anti-Bcl-2 (1 : 1000 dilution), anti-Bax (1 : 1000 dilution), and anti-β-actin (1 : 1000 dilution). Washed the membrane 3 times with TBST, then combined with the appropriate concentration of secondary antibodies, and incubated at room temperature for 2h. The immunoreactive bands were visualized with an enhanced chemiluminescence kit (Thermo Fisher ECL). As mentioned above, NIH Image J software was used for quantitative analysis.

**Immunofluorescence Assay of Fis 1 Expression** Cells cultured on the slides were fixed with 4% paraformaldehyde, washed for 3 times, permeated with 0.5% Triton X-100, sealed with goat serum, incubated with primary antibody (anti-FIS 1) overnight at 4 °C, and then incubated with appropriate secondary antibody. The nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). Fluorescence microscopy was used to observe the records.

**Statistical Analysis** The data were expressed as mean ± standard error of the mean (S.E.M.) and p-values were indicated as detailed in the respective legends. Statistical comparisons were performed (GraphPad prism 6.0 software) using one-way ANOVA with Duncan’s multiple comparisons post-test. Data were considered significantly different with a p-value less than 0.05 (denoted by *p < 0.05, **p < 0.01, ***p < 0.001).

**RESULTS**

**Ampelopsin Inhibited Breast Cancer Cells Growth** To evaluate the effects of Ampelopsin on breast cancer cells growth, two types of cells were used, i.e., triple-negative breast cancer (TNBC) MDA-MB-231 and ER-positive MCF-7 cells. CCK-8 assay was performed to test the effects of Ampelopsin on the proliferation of breast cancer cells. Cells were treated with different concentrations of Ampelopsin for 12 and 24h. As shown in Fig. 1A, Ampelopsin inhibited the proliferations of breast cancer cells. IC_{50} of MDA-MB-231 and MCF-7 cells treated with Ampelopsin for 24h were 41.07 and 50.61 µM, respectively. It can be seen that after treatment with 40 or 60 µM Ampelopsin for 24h, the cell viability of MDA-MB-231 and MCF-7 cells decreased more obviously. In order to further verify the inhibitory effect of Ampelopsin on cell proliferation, a colony formation test was carried out. As the picture shows in Fig. 1B, compared with the control, the number of colonies formed after Ampelopsin treatment was significantly reduced. After 24h of treatment with or without Ampelopsin, observed the changes in cell morphology with a phase microscope. As shown in Fig. 1C, compared with untreated cells, Ampelopsin-treated cells exhibited morphological changes after 24h of treatment, such as cell shrinkage, cell membrane rupture, and cell rounding, which were characteristic features of dying cells. Together, these findings proved that
Ampelopsin inhibited breast cancer cells proliferation.

**Ampelopsin Induced Cell Apoptosis in Breast Cancer Cells** After Ampelopsin treatment, cell apoptosis was checked by Hoechst 33258 nuclear staining. As shown in Fig. 2A, in Ampelopsin-treated cell groups, more cells showed bright blue fluorescence and nuclear pyknosis, indicating cell apoptosis. To determine whether the mitochondria-mediated apoptosis pathways involve in Ampelopsin-induced apoptosis, we examined the expression of apoptosis-related proteins Bcl-2 and Bax in breast cancer cells. The Bax/Bcl-2 ratio is an indicator of cell apoptosis. As shown in Fig. 2B, after treatment with Ampelopsin for 24 h, the level of pro-apoptotic protein Bax increased, while the level of anti-apoptotic protein Bcl-2 significantly decreased. Consistent results were also observed in MCF-7 cells (Fig. 2C).

**Ampelopsin Decreased MMP and Caused ROS Production in Breast Cancer Cells** To confirm the hypothesis that mitochondrial apoptotic pathway may involve in Ampelopsin-induced apoptosis, we used the JC-1 fluorescent probe staining to measure the mitochondrial membrane potential (ΔΨm). The JC-1 monomers show green fluorescence (left panel). While JC-1 aggregates show red fluorescence (middle panel), indicating that the membrane potential is normal. The merged images were presented in the right panel. As shown in Fig. 3A, the red fluorescence decreased when treated with Ampelopsin in both cell lines compared with control cell group, indicating the malfunction of mitochondrial membrane potential. Given that mitochondrial dysfunction is closely related to the promotion of intracellular ROS production, we further investigated the intracellular ROS production. ROS levels (shown as DCFDA fluorescence) increased significantly in the Ampelopsin-treated group compared with the control group (Fig. 3B).

**Ampelopsin Disrupted Mitochondrial Dynamic and Decreased ATP Production** We further characterized mitochondrial morphology using transmission electron microscope in MDA-MB-231 cells. In the cells treated with Ampelopsin, there were condensed mitochondria observed with reduced cristae junctions, dense osmiophilic granular structures in the matrix region, indicating that Ampelopsin disrupted mitochondrial structure of breast cancer cells (Fig. 4A). Meanwhile, Ampelopsin treatment significantly reduced the ATP level in MCF-7 cells (Fig. 4B). Mitochondria are highly dynamic organelles. Morphological and functional changes may happen when there are defects in mitochondrial fusion or fission. In this study, the expression of FIS1 (green) was up-regulated after Ampelopsin treatment in both cell lines (Fig. 4C), indicating

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**Fig. 2. Ampelopsin Induces Apoptosis of MDA-MB-231 Cells and MCF-7 Cell**

A. Hoechst 33258 staining nuclei of MDA-MB-231 cells treated with different concentrations of Ampelopsin for 24 h (Scale bars = 50 μm). The data represents three parallel experiments. B. Western blot analysis (24 h) of MDA-MB-231 cells, and different concentrations of Ampelopsin were used to evaluate Bax and Bcl-2. The protein expression of β-actin was used as standard. The figure on the right shows the ratio of protein density (referred to as dose to control, measured by Image J). Data are expressed as mean ± S.D. of at least three trials. *p < 0.05, **p < 0.01, ***p < 0.001. The far right shows the percentage of the Bax/Bcl-2 ratio. *p < 0.05, **p < 0.01, ***p < 0.001. C. Hoechst 33258 staining nuclei and Western blot analyses of MCF-7 cells treated (24 h) with various concentrations of Ampelopsin (Scale bars = 50 μm). *p < 0.05. (Color figure can be accessed in the online version.)
that Ampelopsin promoted mitochondrial fission and then affected cellular energy metabolism.

**DISCUSSION**

In recent years, a number of cancer chemopreventive phytochemicals have been identified from a variety of dietary plants or herbal medicine. Ampelopsin, which is derived from
the rattan tea, has received an increasing attention due to its promising anticancer properties, especially for osteosarcoma, gastric and breast cancers.\(^{14-16}\) In this study, we discovered that Ampelopsin significantly suppressed the growth of breast cancer cells, suggesting that Ampelopsin possesses anticancer effects either in triple-negative or ER-positive breast cancer. Compared with normal cells, cancer cells are characterized by uncontrolled proliferation, resistance to cell death, invasion

Fig. 4. Effects of Ampelopsin on Mitochondrial Disruption in Breast Cancer Cells

A. Ampelopsin induces the morphological changes of mitochondrial in MDA-MB-231 cells detected by TEM. MDA-MB-231 cells were treated with or without 40 \(\mu\)M Ampelopsin for 24 h (Scale bars = 200 nm). B. ATP levels were measured by ATP assay kit in both cell lines. \(* p < 0.05\). C. Expression of mitochondrial Fission 1 protein FIS1 (green) by immunofluorescence analysis in both cell lines. Nuclei were counterstained with DAPI (Scale bars = 100 \(\mu\)m). (Color figure can be accessed in the online version.)
and metastasis. Previous studies have addressed the possible mechanisms of chemopreventive phytochemicals, including cell cycle arrest, inducing cell death, inhibiting the mammalian target of rapamycin (mTOR) signaling pathway, and increasing intracellular ROS production. Induction of apoptosis is the most prominent property of phytochemicals. However, there are different pathways that lead to cancer cell apoptosis, namely the external pathway through extracellular receptors and the internal pathway through mitochondria. Furthermore, mitochondria are considered to be an important target for cancer treatment due to their roles as integrators of pro-survival and pro-apoptotic pathways. In our study, we focused on the mitochondrial apoptosis pathway through where Ampelopsin exerts anticancer effects.

Firstly, by using two different breast cancer cell lines, we observed the effects of Ampelopsin inducing cell apoptosis. We found that Ampelopsin treatment significantly reduced the proliferation and colony formation in two types of cell lines. Additionally, Ampelopsin-treated cells showed morphological changes consistent with apoptosis.

Next, we investigated the mitochondrial pathway through which Ampelopsin induces apoptosis. With the decreasing of mitochondrial membrane potential, a cascade reaction of apoptosis occurred. Any alteration of ΔΨm results in release of various apoptogenic factors, such as cytochrome c and Smac, into cytoplasm. The released apoptogenic factors bind to apoptotic protease activating factor 1 (Apaf-1), leading to Smac, into cytoplasm. The released apoptogenic factors bind and eventually result in cell death. The release of apoptogenic factors is controlled by the activation of caspase cascade and eventually result in cell death. Caspase-3 activation results in the caspase-dependent cleavage of PARP. Consequently, PARP cleavage leads to formation of DNA ladder, which is a hallmark of apoptosis.

Additionally, Ampelopsin-treated cells showed morphological changes consistent with apoptosis. The cancer cells exposed to Ampelopsin treatment showed morphological changes, including apoptotic bodies, membrane blebbing, and DNA fragmentation.

In conclusion, our study proved that Ampelopsin treatment significantly reduced ΔΨm in both forms of breast cancer cells. In addition, Western blot analysis of Ampelopsin-treated cells showed that Bax was upregulated, while Bcl-2 protein was downregulated at the same time. All these observations indicated that Ampelopsin might promote mitochondrial apoptosis in two breast cancer cell lines through regulating Bax/Bcl-2 expression and simultaneous loss of ΔΨm. These apoptotic results were similar to those previously reported in human thyroid cancer cells induced by Niclosamide.23

Lots of studies have confirmed the direct relationship between ΔΨm loss and ROS production. ROS can cause lipid peroxidation, DNA damage and protein oxidation by inducing the opening of mitochondrial permeability transition pores, and eventually lead to cell apoptosis, which plays an important role in triggering cell apoptosis.

In our study, we observed increased ROS production and ΔΨm loss in breast cancer cells exposed to Ampelopsin treatment. Ampelopsin, together with many other phytochemicals, has dose-dependent and cell-type-dependent antioxidant and pro-oxidant activities. In this study, the cancer cells were exposed to relatively higher levels than in the human bodies. Further studies, which take these variables into account, can help to determine the recommended dietary intake of Ampelopsin from plant foods or specific herbal medicine.

Most cellular ROS are produced by the mitochondria respiratory chain (MRC) due to a small fraction of leaky electrons which escape oxidative phosphorylation (OXPHOS). We proposed that ROS overproduction may result from Ampelopsin-induced mitochondria dysfunction. The present study indicates that Ampelopsin treatment can cause mitochondrial injury evidenced by ATP production, mitochondrial morphological changes and fission protein expression. Usually, ATP depletion serves as an indicator of mitochondrial dysfunction. However, the present study didn’t clarify the target complexes of MRC which are inhibited by Ampelopsin. This is an important issue for future research.

Based on the reduction in ATP production, Ampelopsin-treated cancer cells showed apparent mitochondrial damage, manifested as abnormal morphological changes, including reduced cristae junctions and dense osmiophilic granular structures in the matrix region. These changes may be due to the imbalance in mitochondrial fission and fusion. Mitochondria fission is mainly regulated by Drp1, Fis1 and MFF. Our findings of immunofluorescence analysis demonstrated that Ampelopsin treatment increased mitochondria fission in MDA-MB-231 cells. However, these findings must be interpreted with caution because of the fact that mitochondria are highly dynamic organelle. The role and mechanism of Ampelopsin-induced mitochondrial fusion and fission imbalance in breast cancer remain to be further studied.

At present, the research on the anticancer effect of Ampelopsin is mostly carried out at the cellular level or in animal studies. Research on its clinical application is limited. Nevertheless, it has been reported that Ampelopsin can prevent DOX-induced cardiotoxicity. In addition, Ampelopsin increases the sensitivity of anticancer effect of paclitaxel (PTX) and doxorubicin (DOX) on ovarian cancer cells.

In conclusion, our study proved that Ampelopsin treatment inhibited mitochondrial OXPHOS and reduced ATP production. Ampelopsin-induced mitochondria damage could lead to overproduction of ROS, loss of mitochondria membrane potential and Bax activation, as well as increased mitochondria membrane permeability, which finally induced cell apoptosis. The present study confirmed previous research and contributed additional evidence that mitochondria can be served as an important target for chemopreventive phytochemicals from plant food and herbal medicine.

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