Parthenolide Inhibits the Proliferation of MDA-T32 Papillary Thyroid Carcinoma Cells \textit{in Vitro} and in Mouse Tumor Xenografts and Activates Autophagy and Apoptosis by Downregulation of the Mammalian Target of Rapamycin (mTOR)/PI3K/AKT Signaling Pathway

**Background:**
This study aimed to examine the effects of the sesquiterpene lactone, parthenolide, on migration, autophagy, and apoptosis of MDA-T32 human papillary thyroid carcinoma cells \textit{in vitro} and in mouse tumor xenografts.

**Material/Methods:**
Cell proliferation and viability of MDA-T32 human papillary thyroid carcinoma cells were determined by MTT assay, and cell migration was studied using a transwell assay. Fluorescence microscopy using acridine orange (AO) and ethidium bromide (EB) staining evaluated apoptosis. Transmission electron microscopy was used to study the effects of parthenolide on autophagy, and Western blot examined the levels of autophagy-associated proteins, including Bax, Bcl-2, and LC3-II. Mice (n=10) were injected with 5×10^6 MDA-T32 cells subcutaneously into the left flank, and xenograft tumors were grown for six weeks. Control untreated mice (n=5) were compared with treated mice (n=5) given parthenolide three times per week.

**Results:**
Parthenolide resulted in a dose-dependent reduction in viability and cell migration of MDA-T32 cells, with a half-maximal inhibitory concentration (IC\text{50}) of 12 µM. AO and EB staining showed that parthenolide induced cell apoptosis and electron microscopy identified autophagosomes in MDA-T32 cells. Parthenolide induced increased expression of the autophagocytic proteins, LC3-II and beclin-1, had a dose-dependent inhibitory effect on the mTOR/PI3K/AKT cascade in MDA-T32 cells and inhibited the growth of the mouse xenograft tumors \textit{in vivo}.

**Conclusions:**
Parthenolide inhibited the growth and migration of MDA-T32 human papillary thyroid carcinoma cells \textit{in vitro} and mouse tumor xenografts and activated autophagy and apoptosis by downregulation of the mTOR/PI3K/AKT signaling pathway.

**MeSH Keywords:**
Apoptosis • Autophagy • Cell Migration Assays • Parathyroid Neoplasms

**Corresponding Author:**
Ronghao Sun, e-mail: sporusac@yahoo.com

**Source of support:**
Departmental sources

**Authors’ Contribution:**

- **BCD 1** Chao Li*
- **DEF 1** Yuqiu Zhou*
- **CDF 1** Yongcong Cai
- **BCDE 2** Chunyan Shui
- **BCD 2** Wei Liu
- **BCE 3** Xu Wang
- **AEF 1** Jian Jiang
- **ABC 1** Dingfen Zeng
- **BCE 1** Chunhan Gui
- **AEFG 1** Ronghao Sun

* Chao Li and Yuqiu Zhou contribute to this work equally

1 Department of Head and Neck Surgery, Sichuan Provincial Cancer Hospital, Chengdu, Sichuan, P.R.China
2 Department of Otolaryngology – Head and Neck Surgery, The Affiliated Hospital of Southwest Medical University, Luzhou, Sichuan, P.R. China
3 Graduate School, Chengdu Medical College, Chengdu, Sichuan, P.R. China

**Full-text PDF:**
https://www.medscimonit.com/abstract/index/idArt/915387
Background

Secondary metabolites have been developed by plants to combat the harsh environmental conditions [1]. For several centuries, plant extracts rich in secondary metabolites have been used to treat human disease and, in China, plant extracts are used as traditional medicines. Improved methods in chemical extraction have resulted in the isolation of bioactive molecules from plants and their subsequent use as purified forms of herbal medicines [2]. Sesquiterpene lactones are a large and diverse group of plant metabolites with potent bioactivity [3]. The number of new sesquiterpene lactones from plants is increasing, and currently, there are approximately 500 sesquiterpenes that have been identified [4]. The potential anticancer properties of sesquiterpene lactones have begun to be investigated in clinical trials [5].

Parthenolide is a bioactive sesquiterpene lactone that has the potential to be used in cancer chemotherapy regimens [6]. Parthenolide has been reported to trigger apoptosis in colorectal cancer cells in vitro [7], and to enhance the chemosensitivity of breast and lung cancer cells to paclitaxel [8,9]. In combination with docetaxel, parthenolide has been shown to chemosensitize HBL-100 and MDA-MB-231 breast cancer cells in vitro and reduced tumor spread in vivo in a mouse xenograft model [10]. Radiosensitivity of prostate cancer cells is increased by pretreatment with parthenolide [11]. Also, parthenolide has been reported to halt the growth of cells in a multiple myeloma cell line in vitro [12].

However, the effects of parthenolide have not been previously examined in human thyroid cancer cells in vitro. Thyroid cancers are rare, but their incidence is increasing worldwide, resulting in increased mortality rates for thyroid cancer [12]. The most common type of thyroid cancer is papillary thyroid carcinoma [12]. Late diagnosis and the presence of metastases make advanced thyroid cancer difficult to manage clinically [13]. Surgical excision and chemotherapy are the first-line treatment for early-stage and advanced thyroid cancer, respectively [14]. However, currently available chemotherapy drugs impair the quality of life of the patients due to their adverse toxic effects [15].

Therefore, this study aimed to examine the effects of the sesquiterpene lactone, parthenolide, on migration, autophagy, and apoptosis of MDA-T32 human papillary thyroid carcinoma cells in vitro and in mouse tumor xenografts.

Material and Methods

Cell viability assay

The MTT assay was used to assess the viability and proliferation of MDA-T32 human papillary thyroid carcinoma cells, compared with a normal human thyrocyte cell line. At 70% confluence, the MDA-T32 cells were seeded in 96-well plates and treated with 0–100 μM of parthenolide. After an incubation of 24 hours, the cells were incubated with MTT for 4 h. Then, the media was removed and the colored formazan product was solubilized by 200 μl of dimethyl sulfoxide (DMSO). The viability of the MDA-T32 human papillary thyroid carcinoma cells and the normal human thyrocytes was then determined by measuring absorbance at 570 nm.

Acridine orange (AO) and ethidium bromide (EB) staining

The MDA-T32 cells were cultured in 6-well plates (0.6×10⁶ cells/well) and incubated for 12 h. MDA-T32 cells were treated with parthenolide for 24 h at 37°C, and slides were prepared by placing 10 μl of cell culture onto glass slides. A drop of the AO and EB solution was added and a coverslip was placed on the slides, which were examined using a fluorescence microscope.

Transmission electron microscopy (TEM)

The MDA-T32 human papillary thyroid carcinoma cells were fixed in the solution of 4% glutaraldehyde and 0.05 M sodium cacodylate, postfixed in 1.5% osmium tetroxide (OsO₄), and dehydrated in alcohol. The samples were then embedded in Epon 812 and observed using a Zeiss CEM 902 electron microscope (Zeiss, Oberkochen, Germany).

Cell migration and Invasion assay

Briefly, 1×10⁴ MDA-T32 human papillary thyroid carcinoma cells were maintained in the upper chamber of transwells with 8 μm pores. In the lower chamber, RPMI-1640 medium was added, followed by incubation for 24 h at 37°C. Extracellular matrix gel was used for the cell invasion assay. The non-migrated and non-invasive cells were removed by swabbing. The cells that invaded and migrated to the lower side of the transwell chamber were fixed and stained the crystal violet and observed under the light microscope.

Western blot

The MDA-T32 human papillary thyroid carcinoma cells were harvested and washed with ice-cold phosphate-buffered saline (PBS). RIPA buffer was used for lysis of the cell pellet. A BCA assay was used to determine the protein content of each sample. From each sample, around 30 μg of protein was separated on a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and then transferred to a polyvinylidene fluoride (PVDF) membrane. After being treated with TBS, the membranes were incubated with primary antibody at 4°C and then with secondary antibody. The bands of interest were visualized using enhanced chemiluminescence (ECL) reagent.
Mouse tumor xenografts from MDA-T32 human papillary thyroid carcinoma cells

The National Institutes of Health (NIH) standards for the care and use of laboratory animals were approved by the Sichuan Provincial Cancer Hospital, Chengdu, Sichuan Province, China. Mice were injected with $5 \times 10^6$ MDA-T32 human papillary thyroid carcinoma cells subcutaneously in the left flank. Mice in the control group ($n=5$) were injected intraperitoneally with DMSO (0.1%) in normal saline, and mice in the treated group ($n=5$) were injected with parthenolide diluted with 100 μL normal saline at a dose of 20 mg/kg body weight. Parthenolide treatment and control injections were given three times per week. At the end of six weeks, the mice were euthanized and the xenograft tumors were harvested for further assessment.

**Statistical analysis**

The experiments were performed in triplicate and data were presented as the mean ± standard deviation (SD). Statistical analysis was performed using Student’s t-test. A P-value <0.05 was considered to be statistically significant.

**Results**

**Parthenolide reduced cell viability of MDA-T32 human papillary thyroid carcinoma cells**

The MTT assay examined the effects of parthenolide on cell viability (Figure 1A) of the MDA-T32 human papillary thyroid carcinoma cells. The results showed that parthenolide significantly decreased the proliferation rate of thyroid cancer cells (Figure 1B). The IC$_{50}$ of parthenolide was found to be 12 μM. These effects of parthenolide were found to exhibit a dose-dependent trend, as the proliferation of the MDA-T32 cells decreased with increase in the dose of parthenolide.

**Parthenolide induced apoptosis of the MDA-T32 cells**

Acridine orange (AO) and ethidium bromide (EB) staining showed that parthenolide caused apoptosis of the MDA-T32 human papillary thyroid carcinoma cells, as shown by the increase in AO-stained cells (Figure 2). The induction of apoptosis by parthenolide was accompanied by the increased expression levels of Bax protein and decreased levels of Bcl-2 (Figure 3), which was dose-dependent.

**Parthenolide induced autophagy in the MDA-T32 cells**

Electron microscopy showed that parthenolide treatment of the MDA-T32 cells resulted in the development of autophagosomes (Figure 4). The number of autophagosomes increased as the dose of parthenolide was increased. Parthenolide treatment increased the expression of apoptosis-associated proteins, LC3-II and beclin-1, with no significant effects on LC3-I expression (Figure 5).

**Parthenolide inhibited cell migration of the MDA-T32 cells**

The transwell assay showed that parthenolide inhibited the migration of the MDA-T32 cells at an IC$_{50}$ of 12 μM (Figure 6). The migration rate of the MDA-T32 cells was decreased to 32% compared with the control.

**Parthenolide inhibited cell invasion of the MDA-T32 cells**

The transwell assay showed that parthenolide decreased the invasion of the MDA-T32 cells at an IC$_{50}$ of 12 μM (Figure 7). The invasion rate of the MDA-T32 cells was decreased to 25% compared with the control.
Parthenolide inhibited the mTOR/PI3K/AKT pathway

Parthenolide decreased the phosphorylation of components of the mTOR/PI3K/AKT pathway and included the expression of p-mTOR, p-PI3K, and p-AKT (Figure 8). The total protein levels of mTOR, PI3K, and AKT remained constant, as determined by Western blot analysis.

Parthenolide inhibited MDA-T32 cell xenograft tumor growth

The effects of parthenolide were also assessed on the growth of the MDA-T32 cell mouse tumor xenografts at a dose of 20 mg/kg. Parthenolide suppressed the tumor volume (Figure 9A) and the tumor weight (Figure 9B) of the MDA-T32 cell mouse tumor xenografts.

Figure 2. Fluorescence microscopy using acridine orange (AO) and ethidium bromide (EB) staining evaluated the effects of parthenolide treatment on apoptosis of MDA-T32 human papillary thyroid carcinoma cells. The experiments were performed in triplicate.

Figure 3. Western blot shows the effects of parthenolide on the expression of Bax and Bcl-2 by MDA-T32 human papillary thyroid carcinoma cells. The experiments were performed in triplicate.

Parthenolide inhibited the mTOR/PI3K/AKT pathway

Parthenolide decreased the phosphorylation of components of the mTOR/PI3K/AKT pathway and included the expression of p-mTOR, p-PI3K, and p-AKT (Figure 8). The total protein levels of mTOR, PI3K, and AKT remained constant, as determined by Western blot analysis.

Parthenolide inhibited MDA-T32 cell xenograft tumor growth

The effects of parthenolide were also assessed on the growth of the MDA-T32 cell mouse tumor xenografts at a dose of 20 mg/kg. Parthenolide suppressed the tumor volume (Figure 9A) and the tumor weight (Figure 9B) of the MDA-T32 cell mouse tumor xenografts.
Discussion

Sesquiterpene lactones have shown potential as anticancer agents [16]. The findings of the present study demonstrated the tumor suppressor effects of the sesquiterpene lactone, parthenolide, on MDA-T32 human papillary thyroid carcinoma cells. Parthenolide suppressed the growth of human MDA-T32 thyroid cancer cells with a half-maximal inhibitory concentration (IC$_{50}$) of 12 µM. Previous studies have also shown the potent anticancer effects of parthenolide. Parthenolide has been shown to reduce the proliferation rate of gastric cancer cells in vitro [17]. Parthenolide has also been reported to target NF-kB to suppress the growth of pancreatic cancer cells in vitro [18]. In bladder cancer cells, parthenolide treatment resulted in cell cycle arrest and also increased cell apoptosis [19]. Parthenolide has also been reported to inhibit cell proliferation in human nasopharyngeal carcinoma cells in vitro [20]. The findings of

Figure 4. Electron microscopy shows the effects of parthenolide on the development of autophagosomes in MDA-T32 human papillary thyroid carcinoma cells. The arrows indicate autophagosomes. The experiments were performed in triplicate.

Figure 5. Western blot shows the effects of parthenolide on the expression of LC3-I, LC3-II, and beclin-1 by MDA-T32 human papillary thyroid carcinoma cells. The experiments were performed in triplicate.
the present study have shown that the inhibitory effects of parthenolide on MDA-T32 human papillary thyroid carcinoma cells included increased cell apoptosis, which was also associated with upregulation of Bax and down-regulation of Bcl-2. The findings from previously reported studies support the findings of the present study. Parthenolide has previously been shown to cause apoptosis of HepG2 human liver carcinoma cells [21]. Parthenolide has also been shown to target the PI3K/AKT cascade to suppress the growth of cervical cancer cells in vitro [22]. In the present study, electron microscopy showed that parthenolide treatment caused the development of autophagosomes in the MDA-T32 human papillary thyroid carcinoma cells. The increased expression of LC3-II triggered by parthenolide in the MDA-T32 cells also confirmed that parthenolide induced autophagy in these cells. Previous studies also supported this finding, as parthenolide has been shown to induce autophagy in osteosarcoma cells via the induction of autophagy [23]. Also, parthenolide has been shown to cause autophagy in breast cancer cells in vitro [24], and to suppress the migration and invasion of breast cancer cells [25].

The findings of the present study showed that parthenolide reduced the migration and invasion of MDA-T32 human papillary thyroid carcinoma cells in vitro by downregulating the PI3K/AKT/mTOR signaling pathway. Sesquiterpene lactones, including parthenolide, have become recognized as having anticancer effects via multiple signaling pathways [26]. This study investigated the cell line, MDA-T32, which was isolated in 2014 from a 74-year-old man with papillary thyroid carcinoma and was previously shown to be suitable for the generation of mouse xenograft tumors [27]. In this study, parthenolide was shown to downregulate the PI3K/AKT/mTOR signaling pathway and inhibited the growth of MDA-T32 mouse tumor xenografts in vivo.

**Conclusions**

Parthenolide suppressed the growth of MDA-T32 human papillary thyroid carcinoma cells in vitro by the induction of autophagy and apoptosis. Parthenolide also reduced the migration of MDA-T32 cells. The effects of parthenolide were due to the inhibition of the PI3K/AKT/mTOR signaling pathway.
to downregulation of the PI3K/AKT/mTOR signaling pathway. Further in vivo studies are required to evaluate the effects of parthenolide at different doses and on the different subtypes of human thyroid cancer.

Conflict of interest
None.

References:
1. Newman DJ, Cragg GM: Natural products as sources of new drugs from 1981 to 2014. J Nat Prod, 2016; 79: 629–61
2. Butler MS: Natural products to drugs: Natural product-derived compounds in clinical trials. Nat Prod Rep, 2008; 25(3): 475–516
3. Lee KH, Huang ES, Plantadosi C et al: Cytotoxicity of sesquiterpene lactones. Cancer Res, 1971; 31(11): 1649–54
4. Zhang S, Won YK, Ong CN, Shen HM: Anti-cancer potential of sesquiterpene lactones: Bioactivity and molecular mechanisms. Curr Med Chem Anticancer Agents, 2005; 5(3): 239–49
5. Ghantous A, Gali-Muhtasib H, Vuorela H et al: What made sesquiterpene lactones reach cancer clinical trials? Drug Discov Today, 2010; 15(15–16): 668–78
6. Wu C, Chen F, Rushing JW et al: Antiproliferative activities of parthenolide and golden feverfew extract against three human cancer cell lines. J Med Food, 2006; 9(1): 55–61
7. Zhang S, Ong CN, Shen HM: Critical roles of intracellular thiols and calcium in parthenolide-induced apoptosis in human colorectal cancer cells. Cancer Lett, 2004; 208(2): 143–53
8. Patel NM, Nozaki S, Shortle NH et al: Paclitaxel sensitivity of breast cancer cells with constitutively active NF-kB is enhanced by IkBα super-repressor and parthenolide. Oncogene, 2000; 19(36): 4159–69
9. Gao ZW, Zhang DL, Guo CB: Paclitaxel efficacy is increased by parthenolide via nuclear factor-kappa B pathways in vitro and in vivo human non-small cell lung cancer models. Curr Cancer Drug Targets, 2010; 10(7): 705–15
10. Sweeney CJ, Mehrotra S, Sadaria MR et al: The sesquiterpene lactone parthenolide in combination with docetaxel reduces metastasis and improves survival in a xenograft model of breast cancer. Mol Cancer Ther, 2005; 4(6): 1004–12
11. Sun Y, Clair DK, Xu Y et al: A NADPH oxidase – dependent redox signaling pathway mediates the selective radiosensitization effect of parthenolide in prostate cancer cells. Cancer Res, 2010; 70(7): 2880–90

Figure 8. Western blot shows the effects of parthenolide on the expression of the mTOR/PI3K/AKT pathway by MDA-T32 human papillary thyroid carcinoma cells. The experiments were performed in triplicate.

Figure 9. Effects of parthenolide on mouse xenografts from MDA-T32 human papillary thyroid carcinoma cells. (A) Tumor volume of the MDA-T32 cell mouse tumor xenografts. (B) Tumor weight of the MDA-T32 cell mouse tumor xenografts. The experiments were performed in triplicate (P<0.05).

Indexed in: [Current Contents/Clinical Medicine] [SCI Expanded] [ISI Alerting System] [ISI Journals Master List] [Index Medicus/MEDLINE] [EMBASE/Excerpta Medica] [Chemical Abstracts/CAS]
12. Wu C, Chen F, Rushing JW et al: The natural products parthenolide and andrographolide exhibit anti-cancer stem cell activity in multiple myeloma. Leuk Lymphoma, 2011; 52(6): 1085–97
13. Sipos JA, Mazzaferri EL: Thyroid cancer epidemiology and prognostic variables. Clin Oncol, 2010; 22(6): 395–404
14. De Crevoisier R, Baudin E, Bachelot A et al: Combined treatment of anaplastic thyroid carcinoma with surgery, chemotherapy, and hyperfractionated accelerated external radiotherapy. Int J Radiat Oncol Biol Phys, 2004; 60(4): 1137–43
15. Coates A, Abraham S, Kaye SB et al: On the receiving end – patient perception of the side-effects of cancer chemotherapy. Eur J Cancer Clin Oncol, 1983; 19(2): 203–8
16. Kreuger MR, Grootjans S, Biavatti MW et al: Sesquiterpene lactones as drugs with multiple targets in cancer treatment: focus on parthenolide. Anticancer Drugs, 2012; 23(9): 883–96
17. Sohma I, Fujiwara Y, Sugita Y et al: Parthenolide, an NF-kB inhibitor, suppresses tumor growth and enhances response to chemotherapy in gastric cancer. Cancer Genomics Proteomics, 2011; 8(1): 39–47
18. Yip-Schneider MT, Nakshatri H, Sweeney CJ et al: Parthenolide and sultamic cooperate to mediate growth suppression and inhibit the nuclear factor-kB pathway in pancreatic carcinoma cells. Mol Cancer Ther, 2005; 4(4): 587–94
19. Cheng G, Xie L: Parthenolide induces apoptosis and cell cycle arrest of human 5637 bladder cancer cells in vitro. Molecules, 2011; 16(8): 6758–68
20. Liao K, Xia B, Zhuang QY et al: Parthenolide inhibits cancer stem-like side population of nasopharyngeal carcinoma cells via suppression of the NF-kB/COX-2 pathway. Theranostics, 2015; 5(3): 302–21
21. Sun J, Zhang C, Bao YL et al: Parthenolide-induced apoptosis, autophagy and suppression of proliferation in HepG2 cells. Asian Pac J Cancer Prev, 2014; 15(12): 4897–902
22. Jeyamohan S, Moorthy RK, Kannan MK, Arockiam AI: Parthenolide induces apoptosis and autophagy through the suppression of PI3K/Akt signaling pathway in cervical cancer. Biotechnol Lett, 2016; 38(8): 1251–60
23. Yang C, Yang QQ, Kong QI et al: Parthenolide induces reactive oxygen species-mediated autophagic cell death in human osteosarcoma cells. Cell Physiol Biochem, 2016; 40(1–2): 146–54
24. D’annee A, Carlsen D, Lauricella M et al: Parthenolide generates reactive oxygen species and autophagy in MDA-MB-231 cells. A soluble parthenolide analogue inhibits tumour growth and metastasis in a xenograft model of breast cancer. Cell Death Dis, 2013; 4(10): e891
25. Li CJ, Guo SF, Shi TM: Culture supernatants of breast cancer cell line MDA-MB-231 treated with parthenolide inhibit the proliferation, migration, and lumen formation capacity of human umbilical vein endothelial cells. Chinese Med J, 2012; 125(12): 2195–99
26. Kreuger MR, Grootjans S, Biavatti MW et al: Sesquiterpene lactones as drugs with multiple targets in cancer treatment: focus on parthenolide. Anticancer Drugs, 2012; 23(9): 883–96
27. Henderson YC, Ahn SH, Ryu J et al: Development and characterization of six new human papillary thyroid carcinoma cell lines. J Clin Endocrinol Metab, 2014; 100(2): E243–52