เอกรัตน์ วงษ์พันคำ: ผลของอาร์โทนินอีต่อการตายแบบอะนอยคิสของเซลล์มะเร็งปอด (EFFECTS OF ARTONIN E ON LUNG CANCER CELL ANOIKIS) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ.ภก.ดร.ปิติ จันทร์วรโชติ, 53 หน้า.

อะนอยคิส คือกลไกการตายแบบอะพอพโทซิสซึ่งถูกเหนี่ยวนำโดยการหลุดของเซลล์และอะนอยคิสเป็นกลไกที่ใช้ยับยั้งการแพร่กระจายของเซลล์มะเร็งในร่างกาย พบว่าเซลล์ของมะเร็งปอดจะไม่เกิดอะนอยคิสขึ้นหลังจากเซลล์หลุดออกจากก้อนมะเร็ง ส่งผลให้มีอัตราการแพร่กระจาย และอัตราการตายเนื่องจากมะเร็งเพิ่มสูงขึ้น การวิจัยนี้มีวัตถุประสงค์ เพื่อศึกษาผลของอาร์โทนินอีซึ่งเป็นสารสกัดบริสุทธิ์จากเปลือกลำต้นของขนุนป่า (*Artocarpus gomezianus*) ในการกระตุ้นเซลล์มะเร็งปอดชนิด H460 ให้มีความไวเพิ่มขึ้นในการเกิดอะนอยคิส โดยวัดการรอดชีวิตของเซลล์ด้วยสาร 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) และวัดระดับของโปรตีนซึ่งเกี่ยวข้องด้วยวิธีเวสเทิร์นบลอท ผลการวิจัยพบว่าอาร์โทนินอีสามารถเพิ่มความไวของเซลล์มะเร็งปอดต่อการเกิดอะนอยคิสได้ ซึ่งการตายจะเพิ่มขึ้นตามความเข้มข้นของอาร์โทนินอีผ่านกลไลการลดระดับของโปรตีน myeloid leukemia cell sequence 1 (Mcl-1) โดยไม่มีผลเปลี่ยนแปลงอย่างมีนัยสำคัญทางสถิติต่อระดับของโปรตีน B-cell lymphoma 2 (Bcl-2), Bcl-2-associated X protein (Bax), caveolin-1 (cav-1) และ p53 นอกจากนี้พบว่าอาร์โทนินอีสามารถเพิ่มความไวของเซลล์มะเร็งปอดชนิด A549 และ H292 ต่ออะนอยคิสได้เช่นกัน การวิจัยนี้จึงเป็นการวิจัยชิ้นแรกที่แสดงผลของอาร์โทนินอีต่อการเพิ่มความไวของเซลล์มะเร็งปอดที่หลุดออกจากกลุ่มเซลล์ในการเกิดอะนอยคิส และอาจนำไปสู่การพัฒนาสารดังกล่าวเพื่อใช้เป็นยาในการรักษามะเร็งต่อไปในอนาคต

ภาควิชา เภสัชวิทยาและสรีรวิทยา ลายมือชื่อนิสิต.....

สาขาวิชา เภสัชวิทยา ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

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EKKARAT WONGPANKAM: EFFECTS OF ARTONIN E ON LUNG CANCER CELL ANOIKIS.

ADVISOR: ASST PROF. PITHI CHANVORACHOTE, Ph.D., 53 pp.

Anoikis is apoptosis that is induced by loss of adhesion. Anoikis is recognized as a key inhibitory process of cancer metastasis. Because lung cancer cells possess an ability to resist anoikis resulting in a high rate of metastasis and death, the present study aimed to investigate a possible anoikis-sensitizing effect of artonin E (AE). AE was extracted from bark of *Artocarpus gomezianus*. Anoikis sensitization of AE was investigated in H460 human lung cancer cells. The viable cells were measured by 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) and level of anoikis-related proteins was determined by western blot analysis. AE was shown to enhance H460 cell anoikis in a concentration-dependent manner. We investigated the underlying mechanism of AE and found that AE sensitized the cells by down-regulating antiapoptotic myeloid leukemia cell sequence 1 (Mcl-1) protein but had no significant effect on other proteins including Bcl-2, Bcl-2-associated X protein (Bax), cav-1 and p53. Furthermore, anoikis sensitization of AE was consistently observed in A549 and H292 lung cancer cells. The present study demonstrates a novel activity of AE on lung cancer cell anoikis for the first time which might lead to the development of a new strategy for lung cancer therapy.

Department : Pharmacology and Physiology Student's Signature .....

Field of Study : Pharmacology Advisor's Signature .....

Academic Year : 2012

CHAPTER I

INTRODUCTION

Lung cancer is often found with metastatic tumors at the time of diagnosis (Cheran *et al.*, 2004; Bae *et al.,* 2012). Like other types of cancer, once metastasis occurs, the prognosis of such disease for cure drops dramatically. Therefore strategies that effectively inhibit cancer cell spreading are of interest and benefit the development of new cancer therapy. Among multiple steps of cancer cell metastasis, the process of anoikis, or cell detachment-induced apoptosis, has been recognized as the most crucial cellular mechanism that prevents solid cancer from successfully spreading (Gilmore, 2005; Chiarugi and Giannoni, 2008; Zhong and Rescorla, 2012). In lung cancer, innate and acquired anoikis resistance was frequently found and was linked to a high degree of tumor metastasis and advanced stage of this disease (Wei *et al.*, 2004; Kodama *et al.,* 2005; Li *et al.,* 2008). Many mechanisms of anoikis resistance have been proposed; however, most are regarded as an increase of anti-apoptotic or a decrease of pro-apoptotic proteins of the B-cell lymphoma 2 (Bcl-2) family (Rosen *et al.,* 1998; Coll *et al.,* 2002). An up-regulation of Bcl-2 and myeloid leukemia cell sequence 1 (Mcl-1) proteins was shown to render anoikis resistance in several cancer types (Bondar and McConkey, 2002; Galante *et al*., 2009; Pongrakhananon *et al*., 2010). Since the oligomirization of proapoptotic Bcl-2-associated X protein (Bax) is an essential step generating pores on mitochondria, facilitating the release of cytochrome *c* (Jürgensmeier *et al.,* 1998; Antonsson *et al.,* 2000), it found that upon detachment, Bax is rapidly translocated to the mitochondrial membrane prior to triggering cell anoikis (Valentijn *et al.,* 2003; Valentijn, Zouq, and Gilmore, 2004; Owen *et al.,* 2009). Conversely, the depletion of Bax has been shown to cause anoikis resistance (Gilmore, 2005). Recently, the negative regulatory role of caveolin-1 (cav-1) protein on lung cancer cell anoikis has been reported (Pongrakhananon *et al*., 2010).

Artonin E is a 3-prenylflavone compound extracted from the bark of *Artocarpus gomezianus* Wall. ex Tréc. (Moraceae). Artonin E is also found in other species of genus *Artocarpus* such as *A. scortechinii*, *A. rotunda*, *A. rigida* and *A. altili* (Jagtap and Bapat, 2010). Among 3-prenylflavones isolated from the genus *Artocarpus*, artonin E was well established as exhibiting potential bioactivity including arachidonate 5-lipoxigenase inhibition (Barron and Ibrahim, 1996), antimicrobial activity (Suhartati, Yandri, and Hadi, 2008), antimalarial activity (Boonphong *et al.,* 2007), antituberculosis (Boonphong *et al.* 2007) and cytotoxicity (Boonphong *et al.,* 2007; Suhartati *et al.,* 2008). Since there is no evidence indicating an effect of artonin E in regulation of cancer cell anoikis, the present study aimed to investigate the effect of artonin E on modulating lung carcinoma H460 cell anoikis.

Research Questions

1. Does artonin E affect anoikis response in lung cancer cells?
2. How does artonin E affect pro/anti-apoptotic proteins on lung cancer cell anoikis?
3. How does artonin E affect to cav-1 and p53 in the process of lung cancer cell anoikis?
4. How does the affected protein regulate lung cancer cells to undergo anoikis?
5. Does artonin E effect on anoikis in any other lung cancer cell lines?

Hypothesis

Artonin E induces alteration of pro/anti-apoptotic proteins, cav-1 and p53 results in anoikis of lung cancer cells.

Objectives

1. To investigate the effect of artonin E on lung cancer cell anoikis.
2. To investigate whether pro/anti-apoptotic proteins are affected by artonin E treatment in lung cancer cells.
3. To investigate whether cav-1 and p53 are affected by artonin E.

CHAPTER II

LITERATURE REVIEW

Anoikis and metastasis

Metastatic process is a spread of malignant cells to distant metastatic site and metastasis is the principal event leading to cancer-related death especially lung cancer (Hanahan and Weinberg, 2000). Among multiple steps of cancer cell metastasis (Fig. 1), the process of anoikis, or cell detachment-induced apoptosis (Frisch and Francis, 1994), has been recognized as the most crucial cellular mechanism that prevent solid cancer from successfully spreading (Gilmore, 2005; Chiarugi and Giannoni, 2008; Zhong and Rescorla, 2012). In normal cells, anoikis is initiated by loss of intrigrin-extracellular matrix (ECM) engagement (Frisch and Francis, 1994; Frisch and Ruoslahti, 1997), conversely, innate and acquired anoikis resistance was frequently found in cancer cells (Duxbury *et al.,* 2004). To approach the efficient strategies for metastatic cancer cells, several studies have been made to identify important key involved in anoikis resistance.

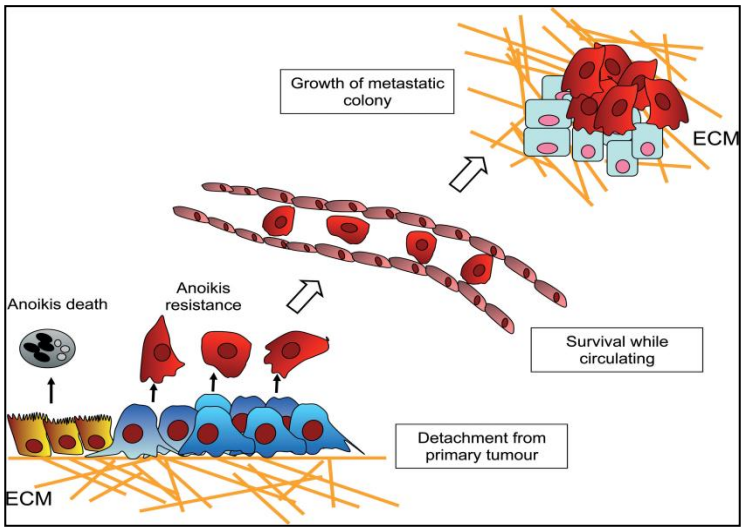


Figure 1. Cancer cells metastasis (Taddei, *et al*., 2009)

Anoikis

Anoikis, or detachment-induced apoptosis, is regulated by anti-apoptotic and pro-apoptotic B-cell lymphoma 2 (Bcl-2) family proteins. In normal cells, anoikis removes displaced cells from incorrect location *via* the alteration of Bcl-2 family proteins and anoikis has been found as a decrease of anti-apoptotic proteins or an increase of pro-apoptotic proteins (Taddei *et al.*, 2012). Bcl-2 family proteins is a crucial protein in anoikis, as well as tumor suppressor, p53 and tumor promoter protein caviolin-1 (cav-1). In detachment cells, cav-1 has shown the upregulation in metastatic cancer cells and the absence of p53 promotes the metastasis of carcinoma cells (Lewis *et al.*, 2005).

1. The anti-apoptotic proteins

Bcl-2 is a member of anti-apoptotic Bcl-2 family proteins. In normal physiological conditions, Bcl-2 prevents apoptosis by heteromerization with Bcl-2-associated X protein (Bax) (Kroemer, 1997; Chen *et al*., 2001; Gazaryan and Brown, 2007). Myeloid cell leukemia sequence 1 (Mcl-1) can also inhibit Bax conformational change at mitochondrial membrane. Anti-apoptotic proteins and Bax interaction prevents oligomerization of Bax into pore and inhibit cytochrome *c* release (Fig. 2) (Jürgensmeier *et al.*, 1998; Antonsson *et al*., 2000).

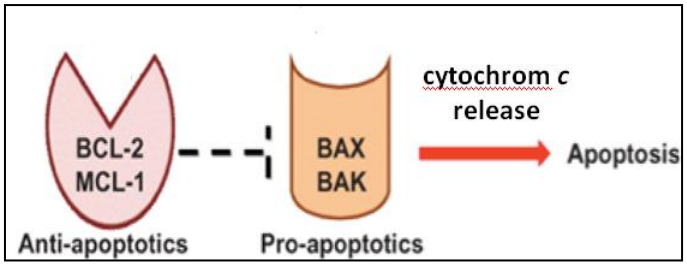


Figure 2. Bcl-2 and Mcl-1 inhibit Bax oligomerization (http://www.cancerdiscovery.aacrjournals.org)

In anoikis studies, Bcl-2 modifying factor (Bmf) that is pro-apoptotic protein showed opposing activity to Bcl-2. Bcl-2 was neutralized in mitochondria by Bmf which was released from myosin V motor complex (Hausmann *et al.*, 2011). The mitochondrial membrane is then generated pore followed by cytochorme *c*

release and activation of caspase (Scorrano and Korsmeyer, 2003). Likewise, the study of MCF-7 breast cancer cells on anoikis showed that anoikis was initiated by the interaction of sequestering Bmf from dynein light chain 2 (DLC2) and Bcl-2 (Puthalakath *et al.,* 2001). Furthermore, Bcl-2 was downregulated in detachment condition (Valentijn *et al.,* 2004; Galante *et al.,* 2009) and it function in interacting and neutralizing pro-apoptotic functions of pro-apoptotic proteins are decreased. Pongrakhananon *et al*. (2010) exhibited the decrease of Bcl-2 protein level during H460 cells detachment and the cells can be sensitized to anoikis by curcumin. These evidences have shown the downregulation of Bcl-2 involve in the anoikis sensitization.

Mcl-1 protein study, the depletion of Mcl-1 which is member of Bcl-2 family proteins resulted in anoikis sensitization of mutant B-RAF melanoma cells (Boisvert-Adamo *et al.,* 2009). Furthermore, degradation of Mcl-1 and induction of Bim promote anoikis (Wood *et al.,* 2007). These studies confirmed that the expression of Mcl-1 affects to cells during detachment.

Bcl-2 and Mcl-1 is the most important key for regulation of anoikis. An downregulation of Bcl-2 and Mcl-1 proteins was exhibited to render anoikis sensitization in several cancer cell types (Bondar and McConkey, 2002; Wood *et al.,* 2007; Galante *et al.,* 2009; Pongrakhananon *et al*., 2010)

2. The pro-apoptotic protein

The pro-apoptotic Bax is one of the members of Bcl-2 family proteins. Bax have been shown to mediate anoikis sensitivity. In previous study, primary mouse mammary epithelial cells and MDCK cells exhibited Bcl-2 levels was not changed in detachment cells while this condition induced rapid tranlocation of Bax from the cytosol to the mitochondria (Pullan *et al.,* 1996; Rytomaa, Lehmann, and Downward, 2000). Woods *et al.* (2007) found that anoikis was initiated by Mcl-1 degradation and Bim induction followed by Bax activation. These data suggested that the multiple step of Bax activation before the initiation of anoikis.

3. The caveolin-1 (cav-1) protein

Cav-1 is major protein component of caveolae. Cav-1 has been associated with oncogenesis. The expression of cav-1 may promote cancer aggressiveness and drug resistance (Williams *et al.*, 2004; Ho *et al.*, 2008; Chunhacha and Chanvorachote, 2012). Conversely, Genetic gene deletion of cav-1 study induced tumorigenesis and lung cancer metastasis (William *et al.*, 2004; Chunhacha and Chanvorachote, 2012).

Cav-1 renders the anoikis *via* cav-1-integrin interaction (Fig. 3). The α-subunit of integrin is bound with cav-1 and can induce the downstream cascades of ERK pathway that is the signal of survival pathway in the adhesion cells (Gilmore, 2005). Cav-1 was downregulated during anoikis (Chunhasha *et al*., 2012, Chanvorachote *et al.*, 2009) and downregulation of cav-1 was resulted from proteosomal ubiquitination pathway (Rungtabnapa *et al*., 2011). Therefore, detached cells were finally sensitized to apoptosis.

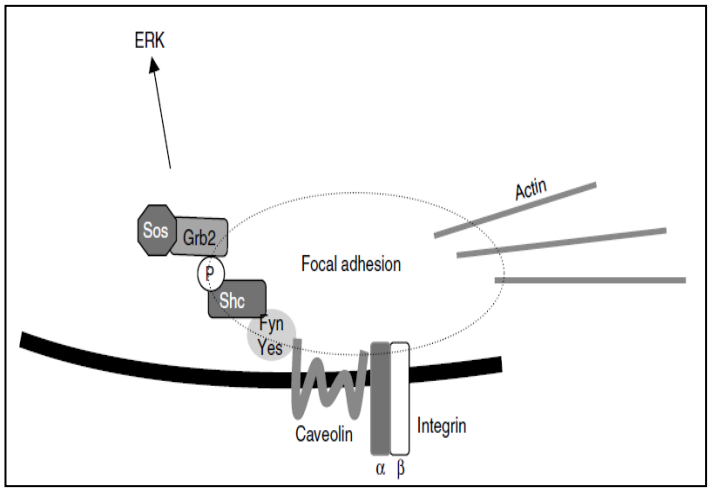


Figure. 3 Caveolin-1 interaction with α-subunit of integrin (Gilmore, 2005)

4. The p53 protein

p53 is a tumor suppressor gene that has an ability to enhance apoptosis. Previously, the data showed that the alteration of fibronectin matrix can both promote the transcription and translation of p53 (Huynh *et al.*, 2002; Dai, 2005; Ghosh, Chen,

and Kapila, 2010). In fibroblast anoikis, p53 regulated Noxa and Puma, BH3-only proteins, at transcriptional level. (Nakano and Vousden, 2001; Shibue *et al.*, 2003) and, thus p53 plays interesting role in anoikis.

Anoikis resistance

In cancer cells, the acquisition of anoikis resistance was frequently found (Duxbury *et al.*, 2004). After detachment from primary site, anoikis resistance cells travel into lymphatic and blood circulating system follow by survive and growth in distant tissue (Fig. 4). To overcome anoikis, malignant cells have altered many molecular proteins. As mention above, Bcl-2 family proteins participate in anoikis resistance, as well as cav-1 and p53. The study about molecular protein mechanism of anoikis resistance is necessary for sensitization cells to anoikis.

In vivo study of gastric cancer cells, MKN74 cells were transfected with Bcl-2 overexpressing plasmid. The upregulation of Bcl-2 led to increase metastasis and extent cell survival. This study showed essential Bcl-2 family role in metastasized cells and anoikis resistance (Yawata *et al.*, 1998). Furthermore, Bcl-2 can play important role in anoikis resistance by the activation of ERK/Bcl-2 pathway which is the prosurvival signal of cells (Galante *et al.*, 2009). Hence, these data verified that the Bcl-2 overexpression induce anoikis resistances (Galante *et al.*, 2009; Pongrakhananon *et al.*, 2010). During anoikis of some study, conversely, Bcl-2 level was not altered (Park *et al.*, 1999).

Mcl-1 protein was shown to render anoikis resistant in several cancer types (Boisvert-Adamo *et al.*, 2009). In anoikis resistance cells, data showed melanoma cell lines rather than melanocytes could control the B-RAF-MEK-extracellular signal-regulated kinase 1/2 pathway for upregulation of Mcl-1 protein. The increase of Mcl-1 provide the anoikis resistance (Boisvert-Adamo *et al.*, 2009) thus, anoikis resistance may result from the presence of Mcl-1 protien.

Cav-1 plays important roles in cells resistance to anoikis as well. Cav-1 stimulated resistance of anoikis in H460 lung carcinoma cells (Pongrakhananon *et al.*, 2010) and was able to enhance Mcl-1 expression via protein-proteins interaction in

cell anoikis (Chunhacha *et al.*, 2012). The several data showed cav-1 can promote cell resistance to anoikis. After detachment, completely anoikis resistant of MCF-7/Cav-1 cells was protected from anoikis suggesting that cav-1 may be protected from anoikis. Recently study, Mcl-1 downregulation was inhibited by cav-1 during detachment condition (Chunhacha *et al.*, 2012). This mechanism may involve with protein-protein interaction. Another study has been demonstrated that the inhibition of detachment-induced p53 activation result in a remarkable and time-dependent manner of cav-1 protein upregulation via the insulin-like growth factor receptor upregulation (Ravid *et al.*, 2005). However, underlying mechanism of cav-1 in anoikis resistance remains unclear.

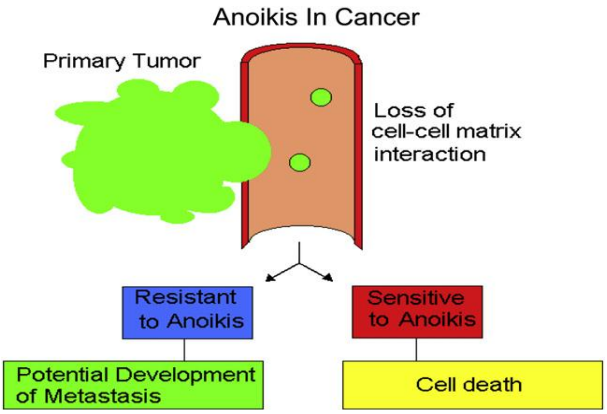


Figure 4. The loss of cell adhesion induces cell death and confers an anoikis signaling in normal cells. Anoikis resistance cells can migration and proliferation in nearby site (Coates, Galante and Bold, 2010).

Artonin E

Artonin E (Fig. 5) is a 3-prenylflavone isolated from bark of *Artocarpus gomezianus* Wall. ex Tréc. (Moraceae) which have known as “Hat-nun” in Thailand. This compound is able to be isolated from some species of genus *Artocarpus* such as *Artocarpus scortechinii, Artocarpus rotunda, Artocarpus rigida, Artocarpus altili* (Jagtap and Bapat, 2010)

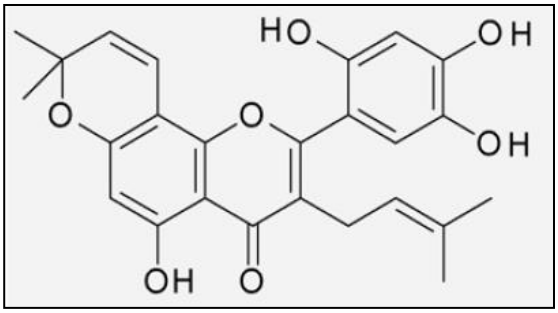


Figure 5. Structure of artonin E

Artonin E which is prenylated at position 3 showed effects on biological pathway such as arachidonate 5-lipoxigenase inhibition activity (Barron and Ibrahim, 1996) and have exhibited potent cytotoxic effect against P-388 leukemia cells (Suhartati *et al.,* 2008).

Cytotoxicity of artonin E

Artonin E added prenyl group that is hydrophobic molecules to the core chemical structure of flavones (*flavus* = yellow) (Fig. 6). Artonin E possessed cytotoxic effects against BC (human breast cancer), KB (Mouth Epidermal Carcinoma) cells and Vero (African green monkey fibroblasts) cell lines (Boonphong *et al.,* 2007). Since artonin E was extracted from the roots of *A. altilis* and could kill BC, KB and Vero cell lines with the IC_{50} of 2.9-14.7µg/ml (Boonphong *et al.,* 2007).

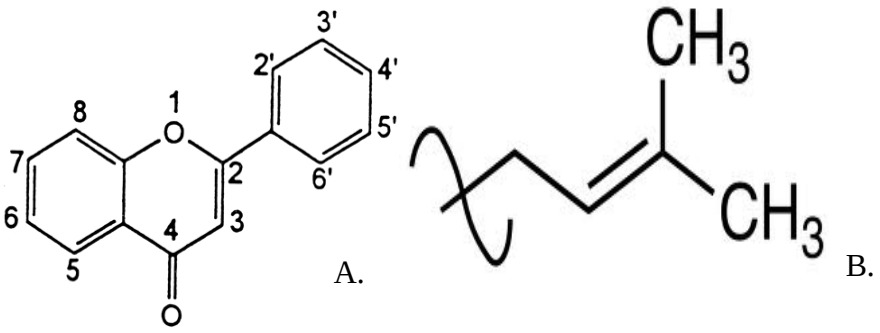


Figure 6. Core structure of flavones (A.) and prenyl group (B.)

Besides, artonin E was well established as exhibiting potential cytotoxic propertity (Boonphong *et al.,* 2007; Suhartati *et al.,* 2008). The extraction of *A. rotunda* root bark of (Hout) includes of prenylflavones especially artonin E that is well-known compound. The artonin E showed significant cytotoxic effect against murine P388 leukemia cells (Suhartati *et al.,* 2001). Likewise, artonin E in extraction from bark of *A. rigida* Blume also had the high cytotoxic effect against P-388 leukemia cells (Suhartati *et al.,* 2008). Seo *et al.* (2003) studied activity of bark isolation of *A. kemand* which is comprised prenylated flavonoids and four phenolic compounds such as artonin E in KB (human oral epidermoid carcinoma). The results had shown cytotoxic effect against KB. This experiment found that artonin E had DNA strand-scission activity. It might be involved with a catechol moiety in ring B. These finding showed that *Artocarpus* extracts, also artonin E, have potential properties to be further researched as new agents of chemotherapy.

CHAPTER III

MATERIALS AND METHODS

Materials

1. Cells

H460, A549 and H292 non-small cell lung carcinoma cells and human kidney 2 (HK2) cells were obtained from the American Type Culture Collection (Manassas, VA, USA).

2. Chemicals and Reagents

Anexin-V FITC kit was obtained from ebioscience (San Diego, USA). Artonin E was obtained from Associate Professor Boonchoo Sritularak. β-actin: rabbit monoclonal IgG, dimethysulfoxide (DMSO), rabbit anti-cav-1 antibody, rabbit anti-Mcl-1 antibody and trypsin was obtained from Cell Signaling (Danvers, MA, USA). 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) reagent was obtained from Sigma Chemical, Inc. (St. Louis, MO, USA). Bradford kit was obtained from Bio-Rad Laboratories (Hercules, CA, USA). Chemiluminescence agent was obtained from Supersignal West Pico (Rockford, IL, USA). Complete Mini cocktail protease inhibitors were obtained from Roche Molecular Biochemicals (Indianapolis, IN, USA). Control plasmid (control shRNA plasmid A), Mcl-1 knockdown plasmid [Mcl-1 short hairpin (sh)RNA plasmid] (shMcl-1), rabbit anti-Bax HRP conjugated, anti-p53 HRP conjugated antibody,was obtained from Santa Cruz biotechnology, Inc. (Santa Cruz, USA). Hoechst 33342 and propidium iodide (PI) were obtained from Molecular Probes, Inc. (Eugene, OR, USA). Horseradish peroxidase-conjugated secondary antibodies, rabbit anti-Bcl-2 antibody were obtained from Abcam (Cambridge, MA, USA). Mcl-1 expression plasmid 25375: pCDNA3.1-hMcl-1 was obtained from Addgene (Cambridge, MA, USA)

3. **Equipments**

These following equipments were use: Analyst/PC densitometry software (Bio-Rad, Hercules, USA). Autopipettes (2-10 µl, 10-100 µl, 20-200 µl and 200-1,000 µl), pipette tips (2-10 µl, 10-100 µl, 20-200 µl and 200-1,000 µl), carbon dioxide incubator, cell culture plate (96-well and 6-well), conical tube (15 ml and 50 ml) (Neptune Equipment Company, Cincinnati, Ohio, USA). CellQuest software (Becton Dickinson, Rutherford, NJ, USA). Laminar flow cabinet, six-well plates tissue culture were coated with 6 mg/ml poly-(2-hydroxyethyl-methacrylate (poly-HEMA) (Sigma-Aldrich, St. Louis, MO, USA). Bottles (100 ml, 250ml, 500 ml and 1,000 ml) (Duran,Wertheim/Main, Germany). Disposable pipette (1ml and 5ml), microplate reader, pH meter and vertex mixer (Perskin Elmer, USA). ELISA reader (Anthros, Durham, NC, USA). Flow cytometer using a 485 nm excitation beam and a 538 band-pass filter (FACsort, Becton Dickinson, Rutherford, NJ, USA). Fluorescent microscope (Olympus IX51 with DP70, Japan). Nitrocellulose membranes were obtained Bio-Rad (Hercules, USA).

Methods

1. Sample preparation

Artonin E was dissolved with ethanol and diluent with complete media to obtain the desired concentrations with less than 0.1% ethanol.

2. Cell culture

H460 and H292 cells were cultured in Roswell Park Memorial Institute medium (RPMI) medium and HK2 and A549 cells were cultured in Dulbecco's Modified Eagle's Medium **(**DMEM). All media were supplemented with 10% of fetal bovine serum (FBS), 2 mmol/l L-glutamine, and 100 units/ml penicillin/streptomycin.

3. Cytotoxicity Assay

Cell survival was determined by XTT colorimetric assay. Briefly, cells were seeded in 96-well plate for 24 h prior to the addition of the test compound. After specific treatment, cells in 96-well plate were incubated with 500 µg/ml of XTT for 4

h at 37°C. The optical density (OD) was spectrophotometrically measured at 570 nm using an ELISA reader. As viable cells could convert yellow XTT to purple formazan by mitochondria reductase, the absorbance of crystal formazan was referred to amount of living cells.

Cell survival was calculated as follow:

$$\text{Percentage of cell survival} = \frac{OD_{570} \text{ of treatment}}{OD_{570} \text{ of control}} \times 100$$

4. Apoptosis and necrosis assay

Apoptosis and necrosis were detected by Hoechst 33342 and PI co-staining. H460 and HK2 cells were incubated with different concentrations of artonin E (0-100 μg/ml) for 24 h in attachment condition. H460, H292 and A549 cells were also detached and suspended for 6 and 12 h in the presence or absence of artonin E at 0-5 μg/ml. Then, cells were stained with 10 μM of the Hoechst and 5 μg/ml PI dyes for 30 min at 37°C and analyzed under a fluorescence microscope using blue filter for Hoechst 33342 and red filter for PI. The apoptotic cells having condensed chromatin and/or fragmented nuclei stained by Hoechst 33342 and PI-positive necrotic cells were visualized and scored under a fluorescence microscope.

5. Anoikis assay

Six-well plate tissue culture were coated with 6 mg/ml poly-HEMA in 95% ethanol and incubated at 37 ^{0}C overnight. Cells in the culture plate were trypsinized into single-cell suspension and then seeded in poly-HEMA-coated plates at a density of 5×10^4 cells/ml. After incubation, 20 μM of XTT reagent was added to the cells for 4 h at 37˚C. The intensity of formazan product was measured at 450 nm using a microplate reader. The cell survival was calculated from optical density readings and represented as percentage to the non-treated control value. The mode of cell death was confirmed by incubating the cells with 10 μg/ml of Hoechst 33342 and visualized under a fluorescent microscope.

6. Annexin-V detection

Cell anoikis were also confirmed by Annexin-V-FITC staining assay using flow cytometry. Cells were collected, re-suspended, and incubated with Annexin V-FITC for 30 min at 37 ^{0}C. Anoikis cells were determined by flow cytometry using 485 nm excitation beam and a 538 band-pass filter. CellQuest software was used for measurement the mean of fluorescence intensity.

7. Cell transfection

The first of all, H460 human lung cancer cells was transfected with Mcl-1-expression plasmid 25375: *p*CDNA3.1-hMcl-1 and Mcl-1-knockdown plasmid [Mcl-1 short hairpin (sh)RNA plasmid] (shMcl-1) and was cultured until ~70% confluence in six-well plate. In the serum free condition, Mcl-1, shRNA-Mcl-1(shMcl-1), control shMcl-1 plasmid A and mock (2 µg) and 15 µl of lipofectamine were used for cell transfection step. After 12 h of transfection, transfected cells were removed medium and replaced 5% FBS medium into plate. After 36 h from the first step of transfection, the trypsinization of cells was performed with 0.03% trypsin. Transfected cells were cultured for 3-4 wks in 75 ml flasks. The protein level of Mcl-1 transfected cells was measured by Western blot method. In each investigation, RPMI 1640 medium without antibiotic agents was used for culture the transfectants for at least two passages before the experiment.

8. Western blot analysis

H460 cells were incubated with different concentrations of artonin E (0-5 µg/ml) for 12 h in detachment condition. Lysis buffer, including 2% Triton X-100, 10 mmol/l Tris–HCl (pH 7.5), Complete Mini cocktail protease inhibitors, 1 mmol/l EDTA, 1% sodium dodecyl sulfate (SDS) and 100 mmol/l NaCl, was used for 30-min incubation with cells, on ice. Lysed cells were centrifuged at 14,000 × *g* for 15 min at 4 $^{\circ}$C, and then supernatant was used for determination of protein content by Bradford method. Protiens (40 µg) were separated by 10% SDS–polyacrylamide gel electrophoresis (SDS–PAGE). After electrophoresis, proteins were transferred using standard procedures to nitrocellulose membranes. The membranes were blocked in

5% skim milk in TBST containing 25 mmol/l Tris–HCl, pH 7.4, 125 mmol/l NaCl, 0.1% Tween 20 for 1 h. Then the membranes were incubated with specific antibody (rabbit anti-Mcl-1 antibody, rabbit anti-cav-1 antibody, rabbit anti-Bcl-2 antibody, rabbit anti-Bax HRP or rabbit anti-p53 HRP conjugated) at 4 $^{\circ}$C overnight, followed by three-times wash of the membranes with TBST for 10 min. The membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 2 h at room temperature. The detection of immune complexes was analyzed by chemiluminescence and quantified by imaging densitometry using analyst/PC densitometry software. Mean densitometric data from independent experiments were normalized to β-actin protein.

9. Statistical Analysis

Mean data from at least three independent experiments were normalized to result in the non-treated control, multiple groups were analyzed by one-way ANOVA and post hoc test (Tukey's test if equal variances were assumed or Dunnett T3's test if equal variance were not assumed) and two groups were analyzed by Student's *t* test at a significance level of $p<0.05$, and presented as the mean $\pm$ S.D.

10. Experimental designs

10.1. Conceptual framework

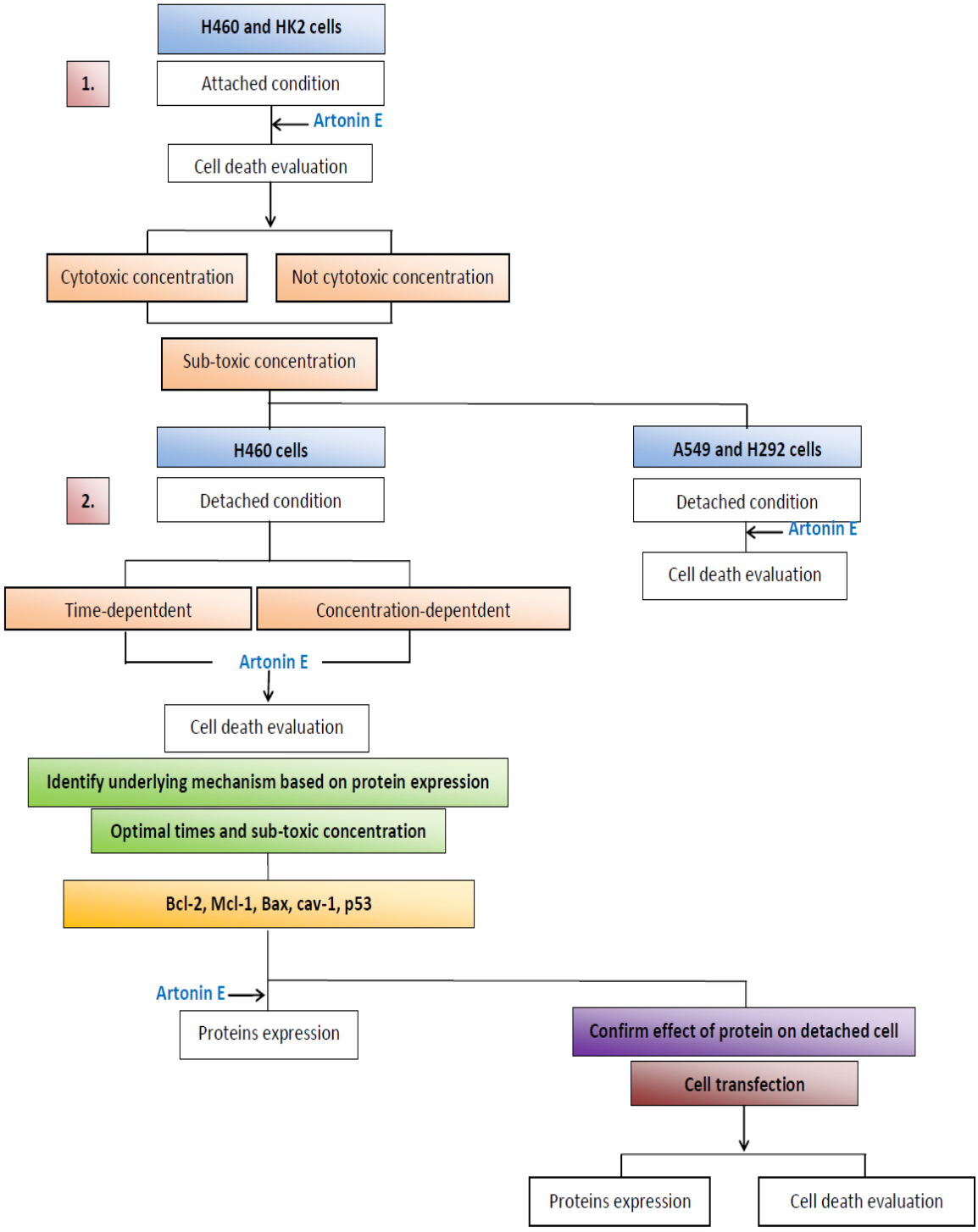


Figure 7. Conceptual frame work of this study

10.2. Cytotoxicity of artonin E

10.2.1. Effect of artonin E on lung cancer H460 cell survival

To study the effect of artonin E on lung cancer cell, we first characterized cytotoxic effect of artonin E in detachment condition of lung cancer H460 cells. Cells were incubated with different concentrations of artonin E (0, 0.5, 1, 5, 10, 50, 100 μg/ml) for 24 h, and cell survival was analyzed by XTT method. To investigate mechanism of cell death with sub-toxic concentration, artonin E-treated H460 cells were incubated with Hoechst 33342 and PI fluorescence dyes and visualized and quantified under fluorescent microscope after 24 h of treatment.

10.2.2. Effect of artonin E on human renal 2 (HK2) cell survival

To study the effect of artonin E on renal cell, we characterized cytotoxic effect of artonin E in HK2 cell. Cells were incubated with different concentrations of artonin E (0, 0.5, 1, 5, 10, 50, 100 μg/ml) for 24 h, and cell survival was analyzed by XTT method. To investigate mechanism of cell death with sub-toxic concentration, artonin E-treated HK2 cells were incubated with Hoechst 33342 and PI fluorescence dyes and visualized and quantified under fluorescent microscope after 24 h of treatment.

10.3. Effect of artonin E on detached H460 cells

To evaluation of anoikis sensitizing effect of artonin E, transfected H460 cells were detached and suspended in the presence or absence of artonin E at 1-5 μg/ml (concentration dependent) and cell survival, apoptosis, and necrosis were analyzed at different times (0-24 h) (time dependent). To investigate mechanism of cell death, detached cells were incubated with Hoechst 33342 and PI fluorescence dyes and visualized and quantified under fluorescent microscope after 12 h of treatment. Also, annexin V staining assay was performed to confirm the apoptosis response of the cells.

10.4. Effect of artonin E on detached H460 cells at protein expression level

To investigate the effect of artonin E on detached condition, detached H460 cells were incubated with artonin E at 1-5 μg/ml and measured Bcl-2, Mcl-1, Bax, cav-1 and p53 protein by standard procedure of western blot analysis as mention above.

10.5. Effect of protein on detached H460 cells

To evaluation of protein effect on detachment condition, H460 cells were transfected with standard procedure, then detached and suspended. Cell apoptosis, and necrosis were analyzed at different times (0-24 h) (time dependent) by Hoechst and PI staining assay. To investigate the protein level of transfected cells, H460 cells were detached at 12 h and measured protein by standard procedure of western blot analysis as mention above.

10.6. Effect of artonin E on lung cancer A549 and H292 cells on detached condition

To study anoikis sensitizing effect of artonin E, A549 and H292 cells were detached in the presence of artonin E at sub-toxic concentrations (1-5 μg/ml) and cell survival, apoptosis, and necrosis were analyzed at 12 h after treatment. To investigate mechanism of cell death, detached cells were incubated with Hoechst 33342 and PI fluorescence dyes and visualized and quantified under fluorescent microscope.

CHAPTER IV

RESULTS

1. Cytotoxicity of artonin E

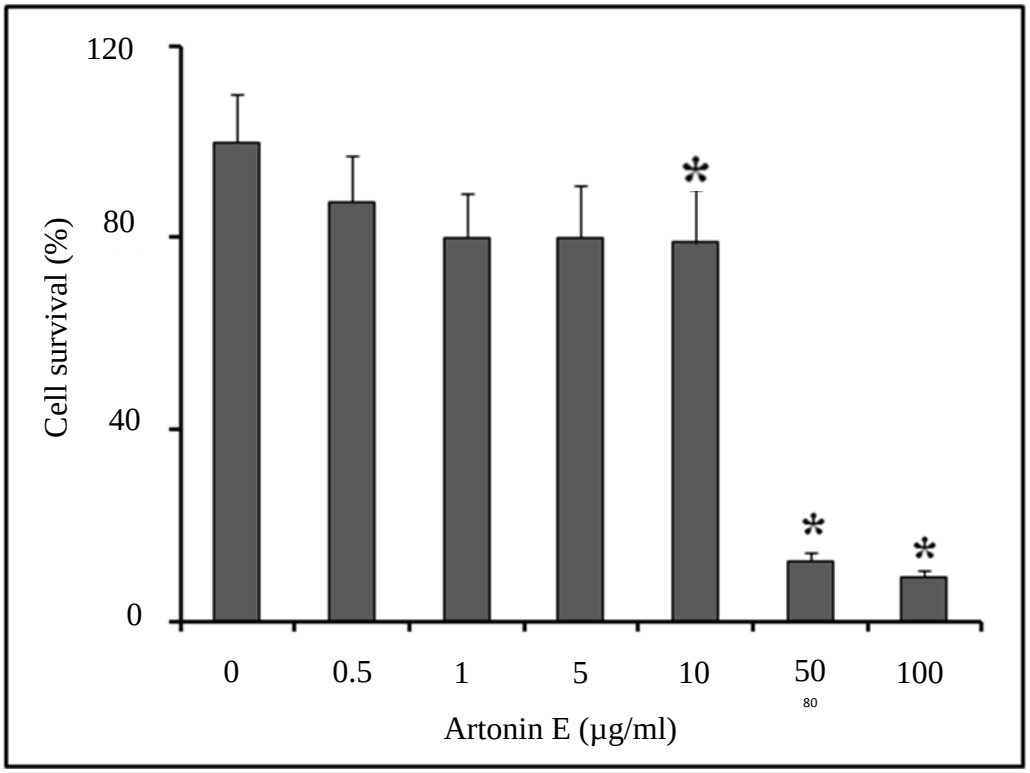
1.1. Effect of Artonin E on lung cancer H460 cell survival

To study the effect of artonin E on lung cancer cell, the investigator first characterized cytotoxic effect of artonin E in lung cancer H460 cells. Attached cells were incubated with different concentrations of artionin E (0, 0.5, 1, 5, 10, 50, 100 μg/ml) for 24 h, and cell survival was analyzed by XTT method.

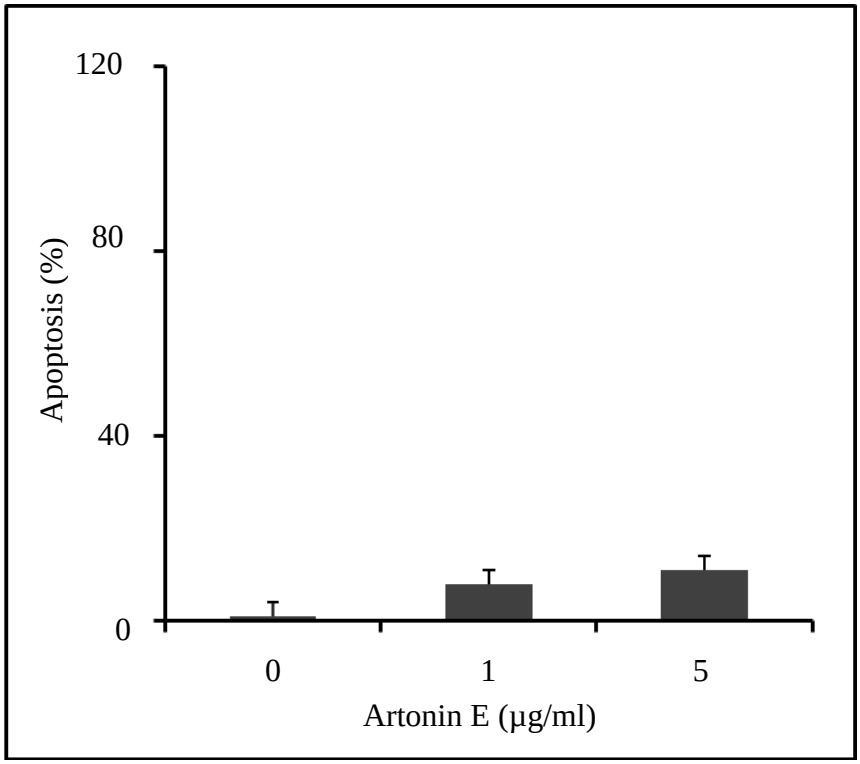
Artonin E at the low concentrations (0-5 μg/ml) caused neither toxic nor proliferative effects on these lung cancer cells. Notably, cytotoxic effect of arotnin E could be observed at the concentrations higher than 10 μg/ml with approximately 80% of the cells remained viable (Fig. 8A).

Concurrent with these findings, Hoechst 33342/ PI staining assay revealed that apoptotic and necrotic cells were not observed in response to artonin E at the concentrations of 0-5 μg/ml. (Fig. 8C)

A


120
80
40
0
Cell survival (%)
*
*
*
0
0.5
1
5
10
50
100
80
Artonin E (μg/ml)

B


120
80
40
0
Apoptosis (%)
0
1
5
Artonin E (μg/ml)

C

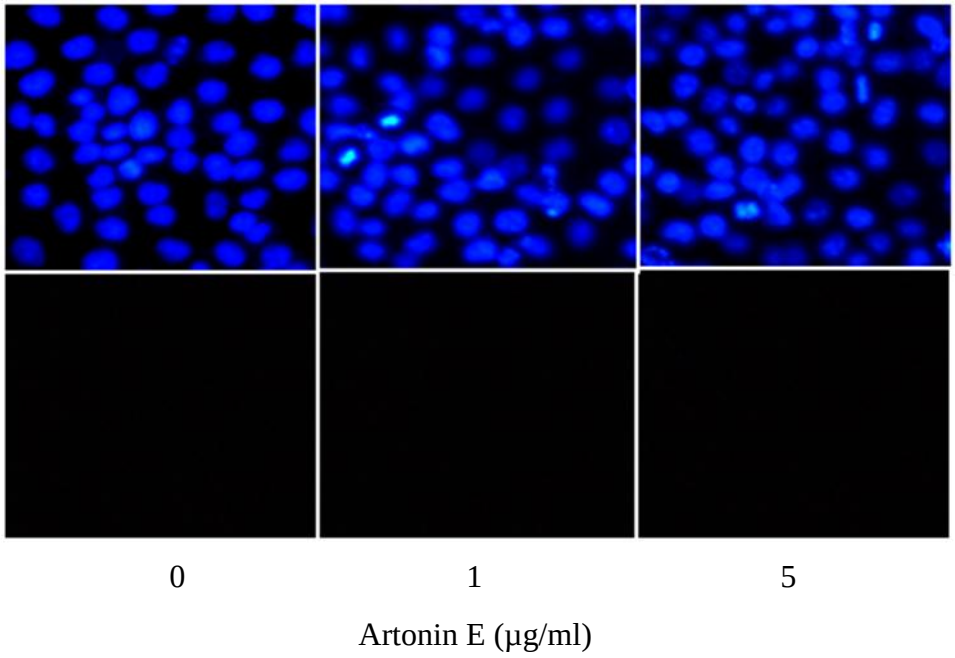


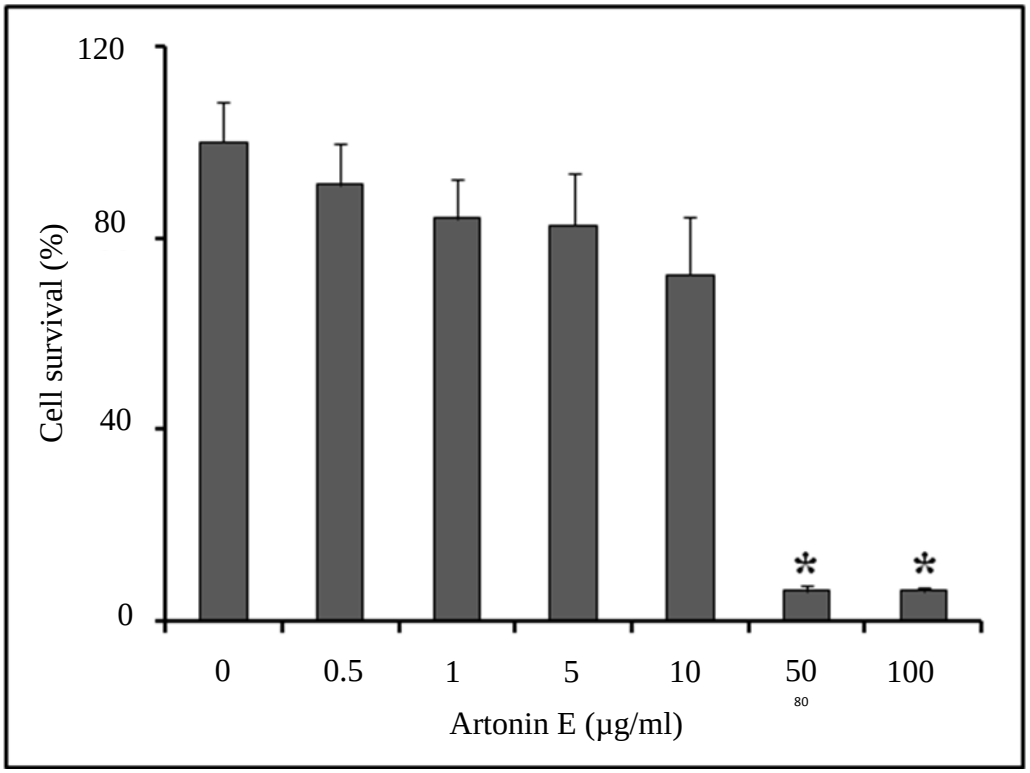
Figure 8. Effect of artonin E on lung carcinoma H460 cell survival. Cells were treated with different concentrations of artonin E (0–100 μg/ml) for 24 h. A, cell survival was measured by XTT assay. Values are means ± S.D. of triplicate experiments. B, Percentage of cell apoptosis was obtained from Hoechst 33342/PI assays. C, Nuclear morphology of Hoechst33342/PI staining cells was captured under fluorescence microscopy. Mean data were analyzed by one-way ANOVA and post hoc test (Dunnett T3's test).

1.2. Effect of Artonin E on human renal HK2 cell survival

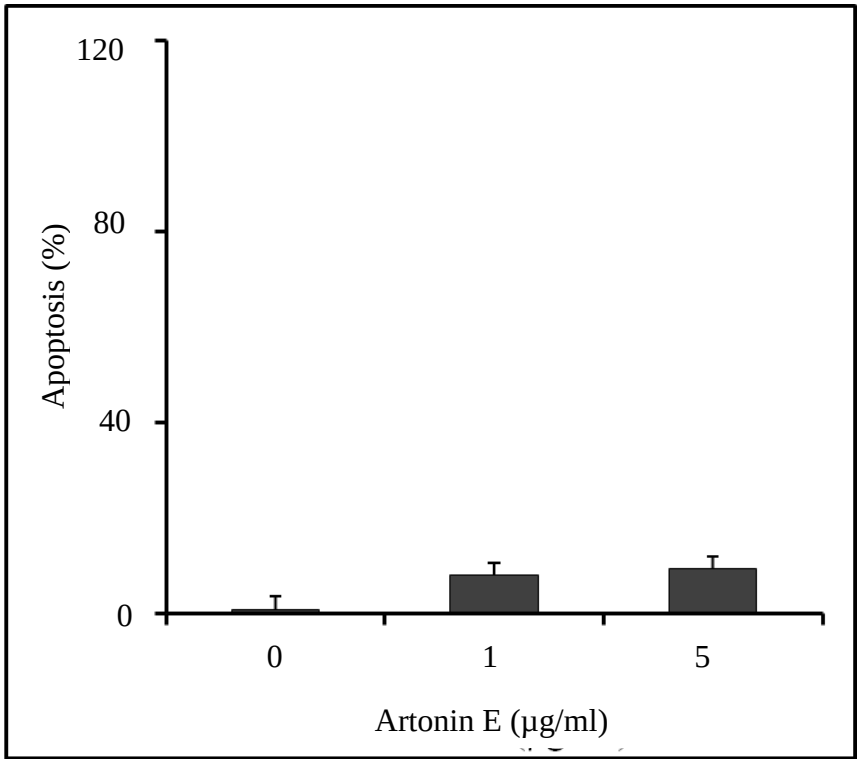
To study the effect of artonin E on human renal cells, the investigator characterized cytotoxic effect of artonin E in HK2 cells. Cells in attached condition were incubated with different concentrations of artionin E (0, 0.5, 1, 5, 10, 50, 100 μg/ml) for 24 h, and viable cells were analyzed by XTT method.

Because major concern for cytotoxic effect of anti-cancer drugs is the cytotoxic to normal cells, the study was performed to test whether artonin E at mentioned concentrations caused significant toxic effect to human renal HK2 cells. The renal cells were treated with artonin E at the concentrations of 0-5 μg/ml and cell survival, apoptosis, and necrosis were evaluated after 24 h. Figure 9A demonstrates that survival of HK2 cells in artonin E treated groups was not significantly altered in comparison to that of non-treated control. Also, we found very limited number of apoptosis and necrosis cells in response to 1-5 μg/ml of artonin E (Fig. 9B and C). These results suggested that artonin E at the concentrations of 1-5 μg/ml exhibited no cytotoxic effect in both neoplastic and normal cells.

A


120
80
40
0
Cell survival (%)
*
*
0
0.5
1
5
10
50
100
80
Artonin E (μg/ml)

B


120
80
40
0
Apoptosis (%)
0
1
5
Artonin E (μg/ml)

C

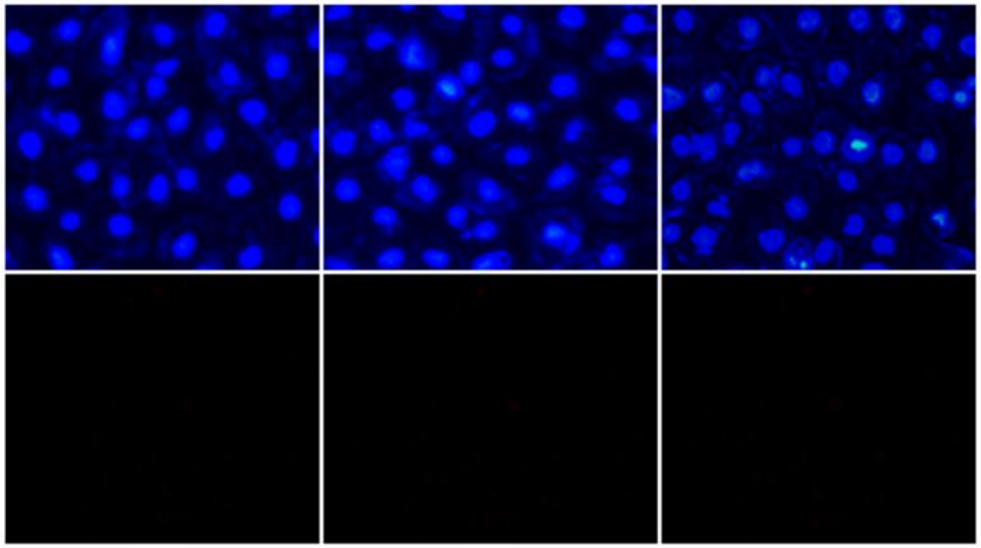


Figure 9. Cytotoxic effect of artonin E in normal kidney HK2 cells. Cells were incubated in the presence or absence of artonin E (0–100 µg/ml) for 24 h. A, cell survival was determined by XTT assay. Values are means ± S.D. of triplicate experiments. B, Percentage of cell apoptosis. C: Nuclear morphology of cells stained with Hoechst 33342 and PI was visualized under fluorescence microscopy. Mean data were analyzed by one-way ANOVA and post hoc test (Dunnett T3's test).

2. Artonin E sensitizes lung carcinoma H460 cells to detachment-induced cell death.

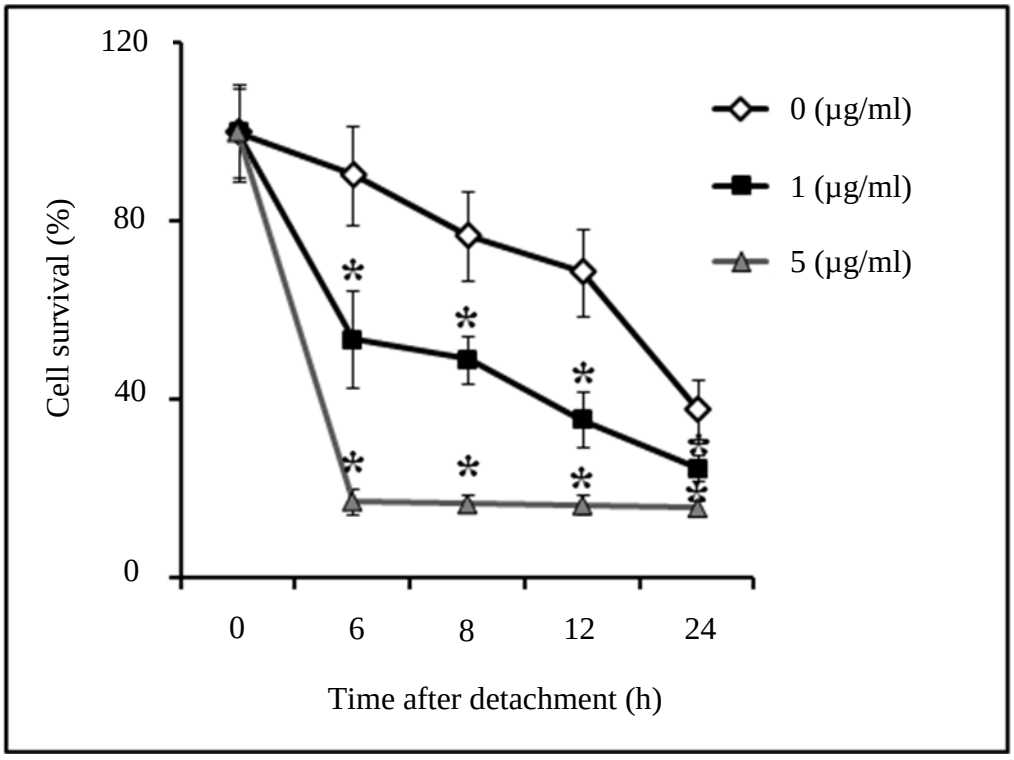
The concentrations of artonin E of 0-5 μg/ml was non-cytotoxic to lung cancer H460 as well as normal renal HK2 cells, Investigator further evaluated anoikis sensitizing effect of artonin E. H460 detached cells were suspended in the presence or absence of artonin E at sub-toxic concentrations and cell survival, apoptosis, and necrosis were analyzed at different times (0-24 h).

Figure 10A shows that H460 cells after detachment exhibited gradually decrease in cell survival over time. Importantly, the treatment artonin E significantly sensitized anoikis of these cells in a concentration-dependent manner. A significant decrease in cell survival in comparison to that of non-treated control was observed as early as 6 h after cell detachment with approximately 50% and 20% of cells remained viable in response to 1 and 5 μg/ml of artonin E, respectively. In control cells, approximately 90% survival of cells was observed at 6 h after detachment and the dramatic decrease in cell survival was first observed at 24 h.

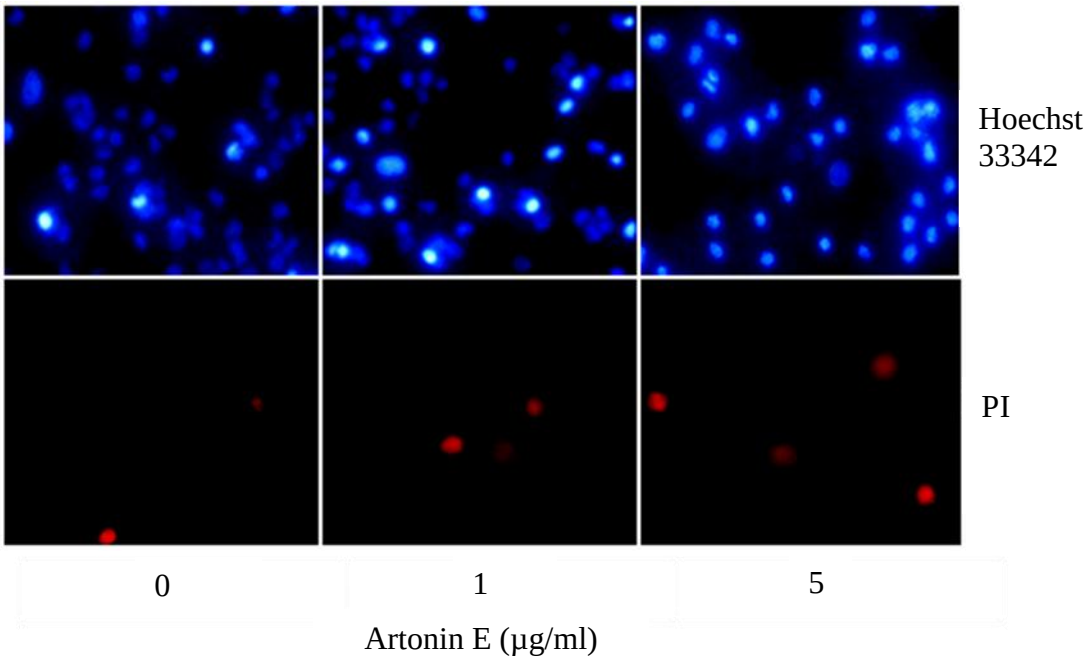
To investigate mechanism of cell death, detached cells were incubated with Hoechst 33342 and PI fluorescence dyes and visualized and quantified under fluorescence microscope. Figure 10B and C show a remarkable increase of intense nuclear fluorescence and chromatin condensation of apoptosis cells. The approximately 70% and 80% apoptosis could be observed in response to 1 and 5 μg/ml artonin E treatment for 12 h, respectively, whereas necrotic cells death was minimal.

Anexin-V staining assay was performed to confirm the apoptosis response of the cells. Consistent with above findings, annexin-V stained cells detected by flow cytometry found to be increase in a concentration-dependent manner in response to artonin E treatment (Fig.10D). These results indicate a potential role of artonin E on anoikis sensitization in human lung cancer cells.

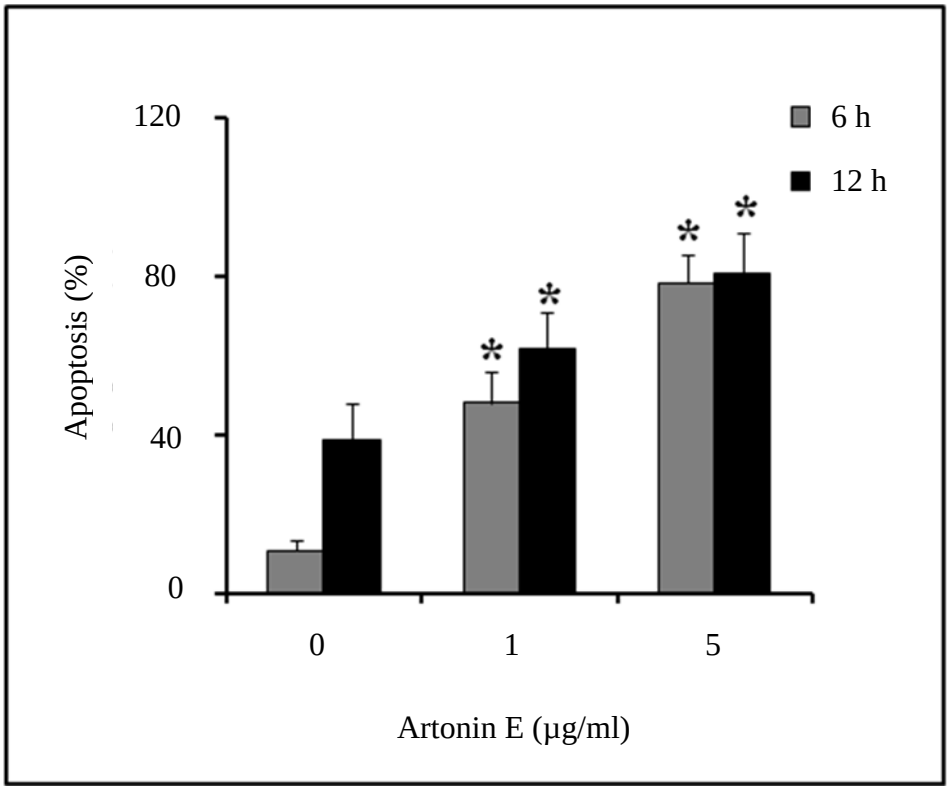
A


120
80
40
0
Cell survival (%)
0 (μg/ml)
1 (μg/ml)
5 (μg/ml)
*
0
6
8
12
24
Time after detachment (h)

B


Hoechst 33342
PI
0
1
5
Artonin E (μg/ml)

C


120
80
40
0
Apoptosis (%)
6 h
12 h
*
*
*
*
0
1
5
Artonin E (μg/ml)

D

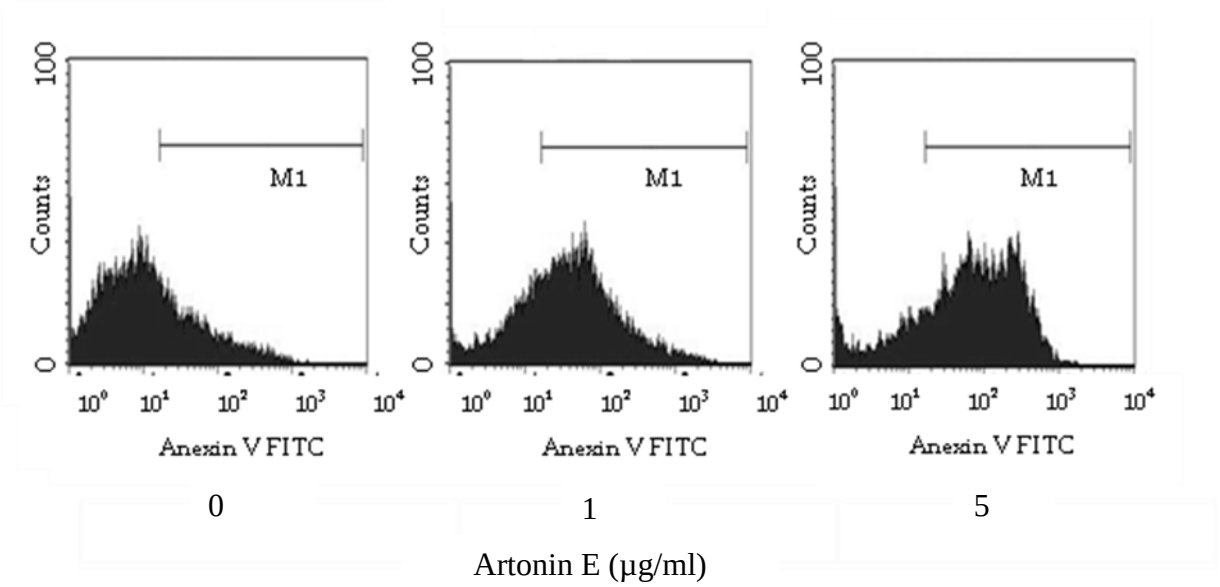

100
0
Counts
M1
10^0 10^1 10^2 10^3 10^4
Anexin V FITC
0
1
5
Artonin E (μg/ml)

Figure 10. Artonin E sensitizes H460 cells to detachment-induced apoptosis. A, Cells were treated with different concentrations of artonin E (0–5 μg/ml) and cell survival was determined by XTT assay at indicated times. Values are means ± S.D. of triplicate experiments, *p<0.05 versus non-treated control. B, Apoptosis and necrosis were detected by Hoechst 33342 and PI staining assay at 6 and 12 h after detachment. C, Nuclear morphology of anoikis cells in response to 12 h of artonin E treatment staining with Hoechst 33342 and PI. D, Apoptosis was evaluated by Annexin V-FITC and flow cytometry at 12 h after detachment. Mean data were analyzed by one-way ANOVA and post hoc test (Dunnett T3's test).

3. Molecular proteins were mediated by Artonin E.

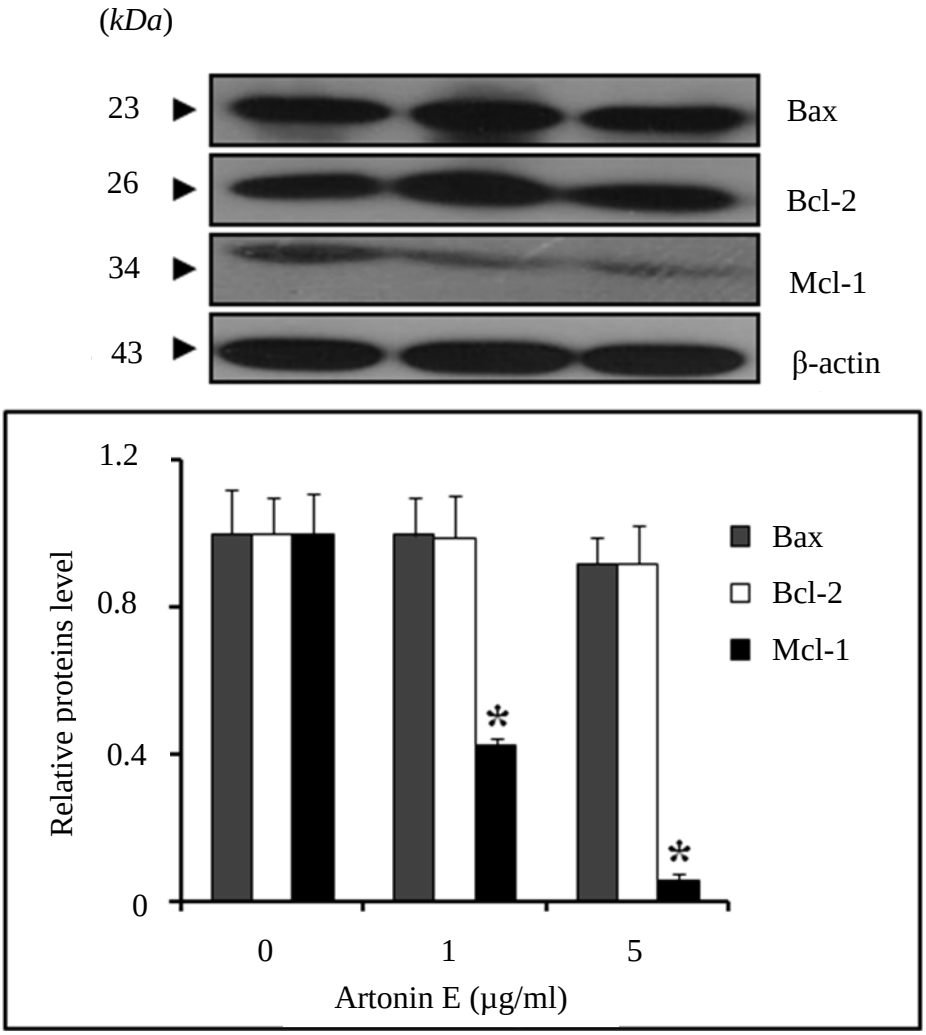
In order to clarify mechanism of artonin E in sensitizing anoikis, effect of artonin E on apoptosis regulating proteins, namely p53, Mcl-1, Bcl-2, and Bax was evaluated.

Detached H460 cells were treated with 1 and 5 μg/ml of artonin E or left untreated for 12 h and expression of mentioned proteins was evaluated by western blot analysis. Figure 11A and B showed that while Bcl-2 and Bax proteins barely were altered.

The Mcl-1 expression significantly decreased in response to artonin E treatment (Fig. 11A).

Since cav-1 protein was shown in many studies that it played an important role on anoikis resistance (Chanvorachote *et al*., 2009; Bonomi *et al*., 2012; Black and Morris, *et al*., 2012; Galante *et al*., 2009), investigator also tested the effect of artonin E on cav-1 expression during anoikis. The artonin E treatment did not cause further reduction of cav-1 (Fig. 11B). These finding suggested that artonin E may, at least in part, sensitized H460 cell anoikis *via* Mcl-1 down-regulation.

A


(kDa)
23
26
34
43
Bax
Bcl-2
Mcl-1
β-actin
1.2
0.8
0.4
0
Relative proteins level
Bax
Bcl-2
Mcl-1
*
*
0
1
5
Artonin E (μg/ml)

B

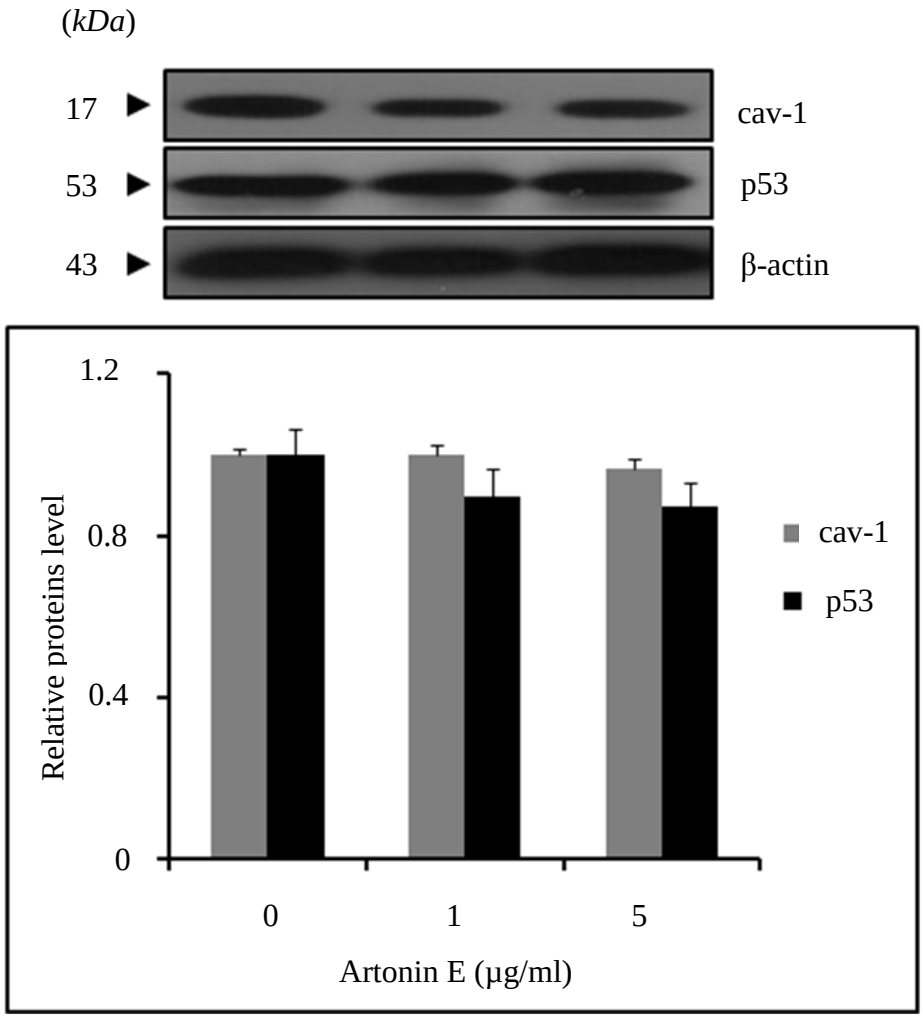


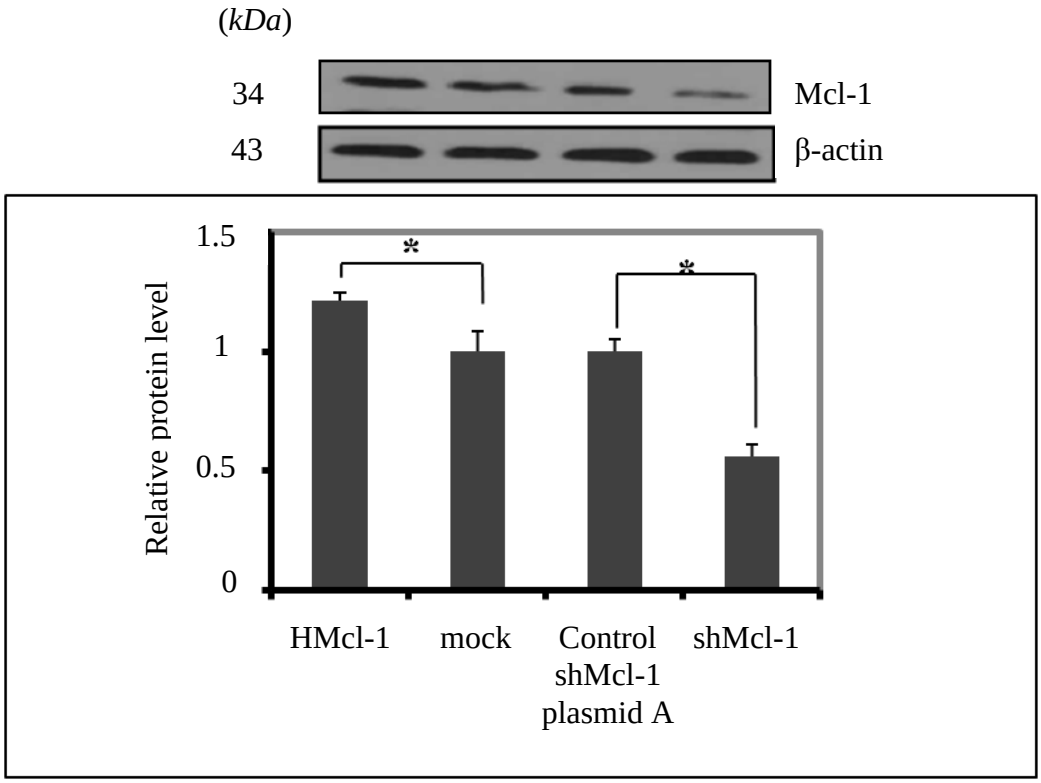
Figure 11. Artonin E sensitizes anoikis via Mcl-1 down-regulation. A, The Bcl-2, Bax and Mcl-1 expressions were detected by Western blotting after 12 h of detachment. B, The p53 and cav-1 expressions were detected by western blot analysis after 12 h of detachment. Values are means ± S.D. (n = 3) **p*<0.05 versus non-treated control. Blots were reported with β-actin antibody to confirm equal loading of the samples**.** Mean data were analyzed by one-way ANOVA and post hoc test (Turkey's test).

4. Mcl-1down-regulation is responsible for anoikis sensitization.

Cells were transfected with Mcl-1 overexpressing, Mcl-1 knock down, or control plasmids. After selection periods, transfectant cells were evaluated for Mcl-1 levels by western blot analysis. Figure 12A shows that the highest Mcl-1 expression was detected in Mcl-1 overexpressing (HMcl-1) cells, while the lowest Mcl-1 was observed in shRNA Mcl-1 transfected (shMcl-1) cells. Then, these transfectants were evaluated for anoikis at indicated times. Results showed that cell survival after detachment declined in a time-dependent manner in all cells.

Corresponding to Mcl-1 levels in these cells, HMcl-1 cells showed the most anoikis resistance characteristic with less than 10 % of apoptosis cells were detected at 12 h after detachment. On the other hand, shMcl-1 cells expressing the lowest level of Mcl-1 protein, was shown to very sensitive to detachment-induced apoptosis (Fig. 12B). These results suggested that Mcl-1 plays an important regulator on anoikis resistance.

A


(kDa)
34
43
Mcl-1
β-actin
Relative protein level
1.5
1
0.5
0
*
*
HMcl-1
mock
Control shMcl-1 plasmid A
shMcl-1

B

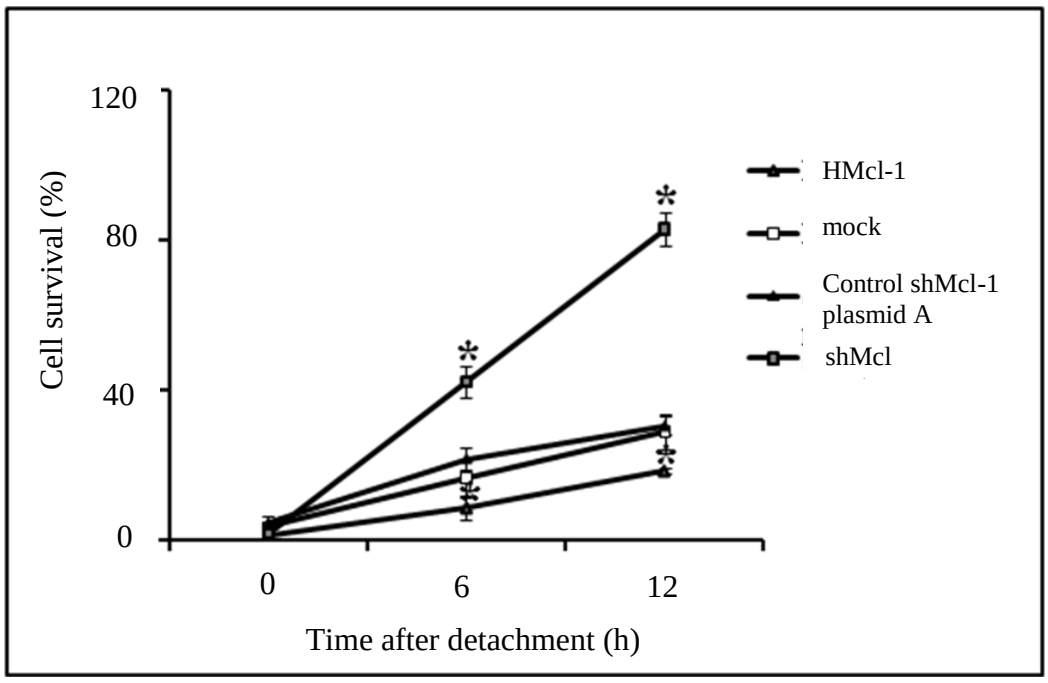

Cell survival (%)
120
80
40
0
0
6
12
Time after detachment (h)
HMcl-1
mock
Control shMcl-1 plasmid A
shMcl
*
*
*
*

Figure 12. Mcl-1 regulates anoikis. A, Mcl-1 mediated anoikis resistance in H460 cells. HMcl-1, Mock, control plasmid A and shMcl-1 cells were cultured in poly-HEMA coated plates with 1 and 5 μg/ml of artonin E and Mcl-1 levels were analyzed by Western blotting after 12 h of detachment. B, Detached Mcl-1 overexpressed, knock down, and control cells were detached for 6, 12 h and apoptotic cells was determined with Hoechst 33342 staining assay. Values are means ± S.D. of triplicate experiments, *p<0.05 versus Mock-transfected control. Mean data were analyzed by Student's t test.

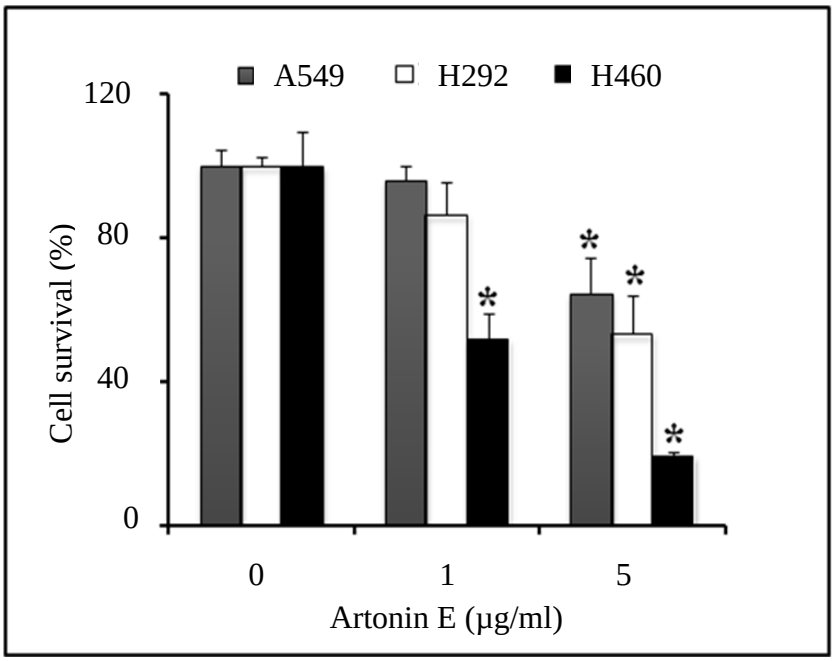
5. Anoikis sensitizing effect of Artonin E in A549 and H292 cells

Having demonstrated anoikis enhancing activity of artonin E in human lung cancer H460 cells, we further confirmed such an effect on other lung cancer cell anoikis models.

After detachment of human lung cancer A549 and H292 cells in the presence or absence of artonin E, cells were similarly evaluated as mentioned above. Figure 13A shows that artonin E significantly decreased cell survival after detachment in both A549 and H292 cells. Even though A549 and H292 exhibited lesser susceptibility to artonin E sensitization, both cells exhibited significant reduced survival in response to at 5 μg/ml of artonin E with approximately 70% of A549 and 50% of H292 cells remained viable in comparison to 10% of H460 cells under the same condition.

Apoptosis cells were also remarkable increased in these cells in response to artonin E treatments, correlated with earlier results (Fig. 13B).

A


A549
H292
H460
120
80
40
0
Cell survival (%)
*
*
*
0
1
5
Artonin E (μg/ml)

B

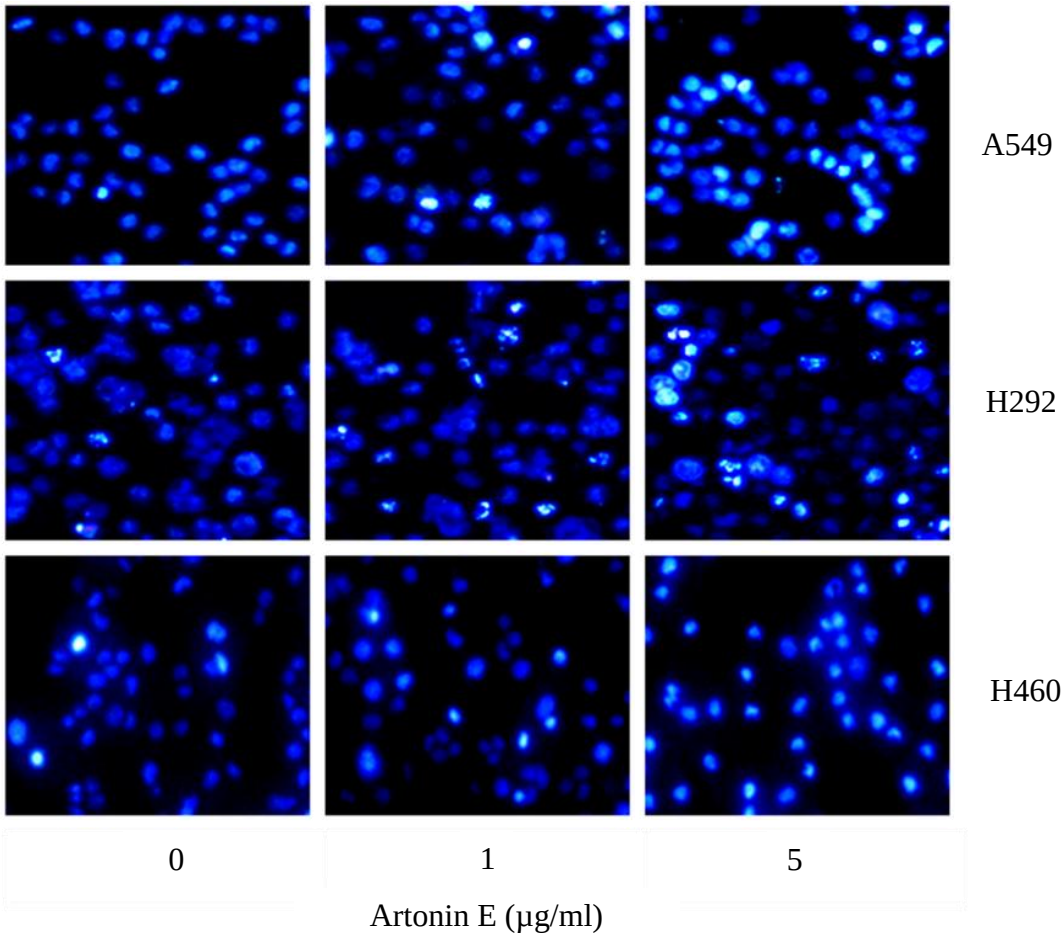

A549
H292
H460
0
1
5
Artonin E (μg/ml)

Figure 13. Effect of artonin E on anoikis of lung cancer A549 and H292 cells. A, A549, H292, and H460 cells were detached and cultured in poly-HEMA coated plates in the presence or absence of artonin E for 12 h and cell survival was determined as described. Values are means ± S.D. (n = 3) *p<0.05 versus non-treated control. B, Apoptosis and necrosis cells were detected by Hoechst 33342 and PI staining assay. Representative photographs are selected from three independent experiments. Mean data were analyzed by one-way ANOVA and post hoc test (Dunnett T3's test).

CHAPTER V

DISCUSSION AND CONCLUSION

Anoikis is a form of apoptosis initiated by the loss or inappropriate contact with the extracellular matrix or surrounding cells (Gilmore, 2005; Kim *et al*., 2012; Simpson *et al*., 2008). Cancer cells either acquire anoikis resistance or possess innate resistant ability could be able to survive after detachment, travel in blood or lymphatic circulations, and establish themselves at distant locations (Kim *et al*., 2012; Nagaprashantha *et al*., 2011). Anoikis resistance enables the cancer cells to spread and establish their secondary tumors (Gilmore, 2005; Kim *et al*., 2012; Simpson *et al*., 2008). So far, mechanisms responsible for anoikis resistance in cancers are intensively investigated and the key proteins which are Mcl-1 and cav-1 were garnered dominant attentions. Overexpression of cav-1, a major protein component in caveolae, was showed to mediate anoikis resistance in lung cancer cells (Chanvorachote *et al.* 2009; Rungtabnapa *et al.* 2011). Also, the evidence further provided the information that expression of such a protein is related to poor prognosis in lung cancers (Yoo *et al*., 2003; Ho *et al*., 2008). Likewise, Mcl-1 was shown to mediated anoikis resistance in melanoma (Boisvert-Adamo *et al.* 2009) as well as lung cancer cells (Li *et al*., 2008; Chunhacha *et al*., 2012). As Mcl-1 classified as an anti-apoptotic protein, it functions in interacting and neutralized pro-apoptotic function of pro-apoptotic proteins preventing the release of cytochrome *c* form mitochondria (Danial and Korsmeyer, 2004). The most important cause of death in lung cancer is metastasis (Tsuya *et al*., 2007; Komatsu *et al*., 2012; Bonomi *et al*., 2012) and such a concept has the development of novel anti-metastasis agents and strategies (Black and Morris, 2012; He *et al*., 2012).

Considering plants as an important source of pharmacologically active compounds, artonin E, a compound isolated from the bark of *Artocarpus gomezianus* was shown to possess several activities such as arachidonate 5-lipoxigenase inhibition, antimicrobial, antimalarial and antituberculosis activity and cytotoxicity (Barron and Ibrahim, 1996; Suhartati *et al*., 2008; Boonphong *et al*., 2007). Also,

artonin E was demonstrated to have cytotoxic effect against leukemia p-388 cell (Suhartati *et al*., 2008). However, there is no evidence indicating the effect of this compound on cancer metastasis. We reported for the first time that artonin E at low concentrations (1-5 µg/ml) which has minimal toxic effect to the normal renal cells exhibited significant anoikis sensitizing activity against lung cancer cells. Besides, major concern for anti-cancer drugs is the cytotoxicity to normal cells. We investigate artonin E with human renal HK2 cells for confirmation of cytotoxic effect. Renal is a major excretion organ to eliminate the metabolites of drugs from human body. Likewise, renal cells have high possibility to exposure the metabolites of drugs and artonin E may be resulting in negative effects on these cells. Thus, HK2 cells were chosen for study the cytotoxicity of artonin E. This study found that the sub-toxic concentration (1-5 µg/ml) of artonin E have no cytotoxic effect to HK2 cells.

Herein, we found that artonin E could be able to decrease cellular level of Mcl-1 during anoikis. The results was supported by the experiment in this study that Mcl-1 down-regulation mediated by shRNA also resulted in the increase sensitivity of the cells to anoikis (Fig. 12B). These results together with our finding that other proteins such as Bcl-2, Bax, Cav-1, and p53 were not affected by the addition of artonin E indicating that artonin E may sensitize the cells to anoikis by reducing cellular Mcl-1. Since Mcl-1 was shown to be an important therapeutic target for the treatment of many cancers (Gores and Kaufmann, 2012; Akgul, 2009), the compound targeting Mcl-1 protein likes artonin E could be of great interest for developing for the use in cancer therapy and related approaches. Furthermore, the variation of cancer cells type was found in many studies. We investigated the effect of artonin E on the others lung cancer cell types. We found that artonin E at the concentration at 5 µg/ml significantly sensitized lung cancer H460, A549, and H292 cells to detachment-induced apoptosis and provide the underlying mechanism.

In summary, the present study has provided the information of artonin E in regulation of anoikis in lung cancer cells. Detailed molecular analysis of artonin E on anoikis resistant cells further provides insight into the mechanism of this compound which may be useful for the development of novel therapeutic strategies for prevention of cancer dissemination.

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APPENDICES

APPENDIX A

TABLES OF EXPERIMENTAL RESULTS

Table 1. The percentage of H460 cell survival in attachment condition was determined by XTT assay after treatment with different concentration of artonin E (concentration dependency). Value represents means ± S.D. of three-independent experiments.

Artonin E (μg/ml)	Cell survival (%)
Control	100.00 ± 10.04
0.5	87.44 ± 9.60
1	80.03 ± 9.39
5	80.09 ± 10.68
10	78.95 ± 11.03
50	12.62 ± 1.97
100	9.25 ± 1.60

Table 2. The percentage of H460 cell apoptosis was stained by Hoechst 33342 staining assay and visualized and scored under fluorescent microscope after treatment with sub-toxic concentration of artonin E (concentration dependency). Value represents means ± S.D. of three-independent experiments

Artonin E (μg/ml)	Apoptosis (%)
Control	2.00 ± 1.00
1	8.67 ± 3.51
5	10.33 ± 4.93

Table 3. The percentage of HK-2 cell survival in attachment condition was determined by XTT assay after treatment with different concentration of artonin E (concentration dependency). Value represents means ± S.D. of three-independent experiments.

Artonin E (µg/ml)	Cell survival (%)
Control	100.00 ± 8.24
0.5	90.98 ± 8.49
1	83.97 ± 8.31
5	82.56 ± 10.83
10	72.26 ± 11.91
50	6.38 ± 1.27
100	6.37 ± 0.66

Table 4. The percentage of HK-2 cell apoptosis was stained by Hoechst 33342 staining assay and visualized and scored under fluorescent microscope after treatment with sub-toxic concentration of artonin E (concentration dependency). Value represents means ± S.D. of three-independent experiments.

Artonin E (µg/ml)	Apoptosis (%)
Control	2.00 ± 1.73
1	8.67 ± 5.51
5	9.33 ± 4.01

Table 5. The percentage of H460 cell survival was determined by XTT assay after treatment with artonin E at different time points (time dependency) and different concentrations (concentration dependency) in detachment condition. Value represents means ± S.D. of three-independent experiments.

Artonin E(µg/ml) / Time (h)	Cell survival (%)		
	0	1	5
0	100.00 ± 10.05	100.00 ± 10.84	100.00 ± 10.74
6	90.40 ± 11.02	53.70 ± 10.92	17.37 ± 2.84
8	76.86 ± 9.87	49.12 ± 5.39	16.74 ± 2.12
12	68.75 ± 9.79	35.60 ± 6.26	16.47 ± 2.24
24	37.95 ± 6.60	24.67 ± 2.89	15.86 ± 1.41

Table 6. The percentage of H460 cell apoptosis was determined by XTT assay after treatment with different concentrations (concentration dependency) and different times (time dependency) of artonin E in detachment condition. Value represents means ± S.D. of three-independent experiments.

Artonin E (µg/ml / Time (h)	Apoptosis (%)		
	0	1	5
6	10.00 ± 2.44	48.11 ± 7.97	78.49 ± 6.78
12	38.52 ± 9.43	61.80 ± 9.07	80.85 ± 9.92

Table 7. The relative of Mcl-1 protein level was determined by Western blot assay after detachment at 12 h. Value represents means ± S.D. of three-independent experiments.

Cell types	Relative protein level
HMcl-1	1.21 ± 0.04
Mock	1.00 ± 0.08
Control shMcl-1 plasmid A	1.00 ± 0.05
shMcl-1	0.55 ± 0.05

Table 8. The percentage of transfected H460 cell apoptosis was stained by Hoechst 33342 staining assay and visualized and scored under fluorescent microscope at different time (time dependency) after detachment condition. Value represents means ± S.D. of three-independent experiments.

Cell types / Time (h)	Apoptosis (%)			
	HMcl-1	Mock	Control shMcl-1 plasmid A	shMcl-1
0	1.31 ± 0.26	3.53 ± 2.78	4.56 ± 0.07	1.85 ± 0.85
6	8.56 ± 2.88	16.66 ± 4.65	21.77 ± 2.78	42.16 ± 4.30
12	18.44 ± 0.85	29.02 ± 4.68	30.63 ± 2.29	82.99 ± 4.29

Table 9. The relative of Mcl-1, Bcl-2, Bax, p53 and cav-1 level was determined by Western blot assay after treatment with different concentrations of artonin E (concentration dependency) at 12 h after detachment. Value represents means ± S.D. of three-independent experiments.

Artonin E (μg/ml) / Protiens	Relative protein level		
	0	1	5
Mcl-1	1.00 ± 0.11	0.43 ± 0.01	0.05 ± 0.02
Bcl-2	1.00 ± 0.10	0.99 ± 0.11	0.92 ± 0.11
Bax	1.00 ± 0.12	0.99 ± 0.10	0.91 ± 0.07
p53	1.00 ± 0.07	0.90 ± 0.07	0.87 ± 0.06
cav-1	1.00 ± 0.05	0.99 ± 0.02	0.97 ± 0.02

Table 10. The percentage of A549, H292 and H460 cell survival was determined by XTT assay after treatment with different concentrations of artonin E (concentration dependency) at12 h in detachment condition. Value represents means ± S.D. of three-independent experiments.

Cell types / Artonin E (μg/ml)	Cell survival (%)		
	A549	H292	H460
0	100.00 ± 4.30	100.00 ± 2.25	100.00 ± 9.24
1	95.79 ± 3.89	86.43 ± 9.07	51.98 ± 6.71
5	64.32 ± 10.27	53.14 ± 10.67	16.39 ± 0.85

APPENDIX B

CELL LINES

1. H460 cell line

In 1982, A.F. Gazdar and colleagues collected the large cell cancer of pleural fluid from the patient. The H460 cell line shows no gross structural DNA abnormalities, and exhibits easily detectable p53 mRNA at levels comparable to normal lung tissue. For stained cells, the cells express positively for vimentin and keratin but are negative for neurofilament triplet protein (American Type Culture Collection; ATCC).

2. A549 cell line

In 1972, D.J Giard and associates developed A549 cell line from the removal and culturing of malignant lung tissue in metastatic cancer of 58-year-old caucausian male. The A549 cell line is human alveolar basal epithelial cells. For immunoperoxidase staining technique, the cells are positive for keratin. The A549 cells contain a high level of desaturated fatty acids and can produce lecithin (American Type Culture Collection; ATCC).

3. H292 cell line

32-year-old black female was collected a cervical node metastasis of a pulmonary mucoepidermoid carcinoma and cultured until development to H292 cell line. Cells exhibit positively mucoepidermoid characteristics in culture and support replication of Hepatitis B virus. Some of the cells contain numerous small mucin-containing granules and are L-DOPA decarboxylase negative. The cells are mucicarmine positive and also stain positively for keratin and vimentin but negative for neurofilament triplet protein (American Type Culture Collection; ATCC).

4. HK2 cell line

HK-2 (human kidney 2) is a proximal tubular cell line derived from normal kidney. The cells were immortalized by transduction with human papilloma virus 16 (HPV-16) E6/E7 genes. HK-2 cells retain functional characteristics of proximal tubular epithelium such as Na+ dependent / phlorizin sensitive sugar transport and adenylate cyclase responsiveness to parathyroid, but not to antidiuretic hormone. The cells are capable of gluconeogenesis as evidenced by their ability to make and store glycogen. HK-2 cells are anchorage dependence. The cells will not grow in methylcellulose, soft agar or suspension (American Type Culture Collection; ATCC).

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VITA

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