Artonin E Mediates MCL1 Down-regulation and Sensitizes Lung Cancer Cells to Anoikis

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Abstract. *Background: Anoikis, or detachment-induced apoptosis, is recognized as a key inhibitory process of cancer metastasis. Since lung cancer cells possess an ability to resist anoikis, resulting in a high rate of metastasis and death, the present study aimed to investigate the possible anoikis-sensitizing effect of artonin E (AE). Materials and Methods: AE was extracted from bark of Artocarpus gomezianus. Anoikis sensitization of AE was investigated in H460, A549 and H292 human lung cancer cells. The level of anoikis-related proteins was determined by western blot analysis and viable cells were measured by the 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) method. Results: AE was shown to enhance anoikis of H460 cells in a dose-dependent manner. We investigated the underlying mechanisms of AE on anoikis sensitization and found that AE sensitized the cells by down-regulating the anti-apoptotic myeloid leukemia cell sequence-1 (MCL1) protein but had no significant effect on other proteins of the B-cell lymphoma-2 (BCL2) family, including BCL2 and BCL2-associated X protein (BAX). Anoikis sensitization of AE was consistently observed in A549 and H292 lung cancer cells. Conclusion: 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.*

Lung cancer is often found with metastatic tumors at the time of diagnosis (1, 2). As in other types of cancer, once metastasis occurs, the prognosis of such disease drops dramatically. Therefore strategies that effectively inhibit cancer cell spreading are of interest and benefit the development of new cancer therapies. 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 (3-7). In lung cancer, innate and acquired anoikis resistance was frequently found and has been linked to a high degree of tumor metastasis and an advanced stage of this disease (8-10). 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 (BCL2) family (7, 11-14). An up-regulation of BCL2 and myeloid leukemia cell sequence-1 (MCL1) proteins was shown to render anoikis resistance in several cancer types (15-18). Since the oligomirization of pro-apoptotic BCL2-associated X protein (BAX) is an essential step generating pores on mitochondria, facilitating the release of cytochrome *c* (19, 20), it has been found that upon detachment, BAX is rapidly translocated to the mitochondrial membrane prior to triggering cell anoikis (21-23). Conversely, the depletion of BAX has been shown to cause anoikis resistance (5). Recently, the negative regulatory role of caveolin-1 (CAV1) protein on lung cancer cell anoikis has been reported (16).

Artonin E (AE) is a 3-prenylflavone compound extracted from the bark of *Artocarpus gomezianus* Wall. ex Tréc. (Moraceae) (Figure 1). AE is also found in other species of genus *Artocarpus* such as *A. scortechinii*, *A. rotunda*, *A. rigida* and *A. altili* (24). Among 3-prenylflavones isolated from the genus *Artocarpus*, AE was well-established as exhibiting potential pharmacological properties including arachidonate 5-lipoxigenase inhibition (25), antimicrobial (26), antimalarial (27), antituberculosis (27) and cytotoxicity (26, 27). Since there is no evidence indicating an effect of AE in the regulation of cancer cell anoikis, the present study aimed to investigate the effects of AE on modulating lung carcinoma H460 cell anoikis.

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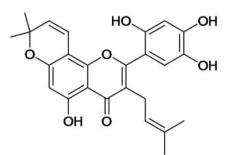
Figure 1. *Chemical structure of artonin E.*

Materials and Methods

Cell culture and reagents. 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). H460 and H292 cells were cultured in Roswell Park Memorial Institute medium (RPMI) medium and human kidney 2 (HK2), respectively and A549 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM). All media were supplemented with 10% fetal bovine serum (FBS), 2 mmol/l L-glutamine, and 100 units/ml penicillin/streptomycin. Propidium iodide (PI), and Hoechst 33342 were obtained from Molecular Probes, Inc. (Eugene, OR, USA). Rabbit anti-MCL1 antibody was purchased from Cell Signaling (Danvers, MA, USA).

Anoikis assay. Six-well tissue culture plates were coated with 6 mg/ml poly-(2-hydroxyethyl-methacrylate) (poly-HEMA) (Sigma–Aldrich, St. Louis, MO, USA) in 95% ethanol and incubated at 37°C overnight. Cells in the culture plate were trypsinized into a 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 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) reagent (Sigma–Aldrich, St. Louis, MO, USA) was added to the cells for 4 h at 37°C. The intensity of the formazan product was determined at 450 nm using a microplate reader. Cell viability was calculated from the optical density (OD) readings and is represented as a percentage to that of the non-treated control value. The mode of cell death was confirmed by incubating the cells with 10 μg/ml of Hoechst 33342 and visualization under a fluorescence microscope (Olympus IX51 with DP70) (Olympus, Japan).

Annexin-V detection by flow cytometry. Cell anoikis was confirmed by annexin V-fluorescein isothiocyanate (FITC) staining assay and flow cytometry. Cells were collected, re-suspended, and incubated with annexin V-FITC for 30 min at 37°C. Cells were scored by flow cytometry using a 485 nm excitation beam and a 538 band-pass filter (FACsort, Becton Dickinson, Rutherford, NJ, USA). The mean fluorescence intensity was quantified by the CellQuest software (Becton Dickinson).

Hoechst 33342 and PI staining. Apoptotic and necrotic cell death was determined by Hoechst 33342 and PI co-staining H460 and HK2 cells were incubated with different concentrations of AE (0-100 μg/ml) for 24 h in attachment condition and H460, H292 and A549 cells were also detached and suspended for 6 and 12 h in the presence or absence of AE at 0-5 μg/ml. Then, cells were incubated with 10 μM of the Hoechst and 5 μg/ml PI for 30 min at 37°C. Apoptotic cells having condensed chromatin and/or fragmented nuclei and PI-positive necrotic cells were visualized and scored under a fluorescence microscope (Olympus IX51 with DP70) (Olympus, Japan).

Western blot analysis. H460 cells were incubated with different concentrations of AE (0-5 μg/ml) for 12 h in detachment conditions. Cells were then incubated with lysis buffer containing 2% Triton X-100, 1% sodium dodecyl sulfate (SDS), 100 mmol/l NaCl, 10 mmol/l Tris–HCl (pH 7.5), 1 mmol/l EDTA, and Complete Mini cocktail protease inhibitors (Roche Molecular Biochemicals, Indianapolis, IN, USA) for 30 min on ice. After insoluble debris was pelleted by centrifuging at 14,000 ×*g* for 15 min at 4°C, the supernatant was collected and the protein content was determined using the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA). Proteins (40 μg) were resolved on 10% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and then transferred onto nitrocellulose membranes (Bio-Rad) using standard procedures. The membranes were blocked in 5% skimmed milk in TBST (25 mmol/l Tris–HCl, pH 7.4, 125 mmol/l NaCl, 0.1% Tween 20) for 1 h, followed by incubation with rabbit anti-MCL1 antibody, (Cell Signaling, Danvers, MA, USA), rabbit anti-CAV1 antibody, rabbit anti-BCL2 antibody (Abcam, Cambridge, MA, USA), rabbit anti-BAX HRP and rabbit anti-p53 HRP conjugated antibody (Santa Cruz biotechnology, Inc. Santa Cruz, USA) at 4°C overnight. Membranes were washed three times with TBST for 10 min, followed by incubation with horseradish peroxidase-conjugated secondary antibodies (Abcam, Cambridge, MA, USA) for 2 h at room temperature. The immune complexes were detected by chemiluminescence (Supersignal West Pico; Pierce, Rockford, IL, USA) and quantified by imaging densitometry using the analyst/PC densitometry software (Bio-Rad). Mean densitometric data from independent experiments were normalized to β-actin protein.

Statistical analysis. Mean data from at least three independent experiments were normalized to values for the non-treated controls, analyzed by one-way ANOVA at a significance level of $p < 0.05$, and are presented as the mean±S.D.

Results

Effect of AE on H460 lung cancer cell viability. In order to study the effect of AE on lung cancer cell anoikis, we first characterized the cytotoxic effect of AE on H460 lung cancer cells. Cells were incubated with different concentrations of AE (0-100 μg/ml) for 24 h, and cell viability was followingly analyzed. Figure 2A shows that AE at low doses (0-5 μg/ml) caused neither toxic nor proliferative effects on these lung cancer cells. Notably, cytotoxic effect of AE was observed at concentrations higher than 10 μg/ml, with approximately 80% of the cells remaining viable (Figure 2A). Concurrent with these findings, Hoechst 33342/ PI staining assay revealed that apoptotic and necrotic cells were not observed in response to AE at the concentrations of 0-5 μg/ml (Figure 2B and C).
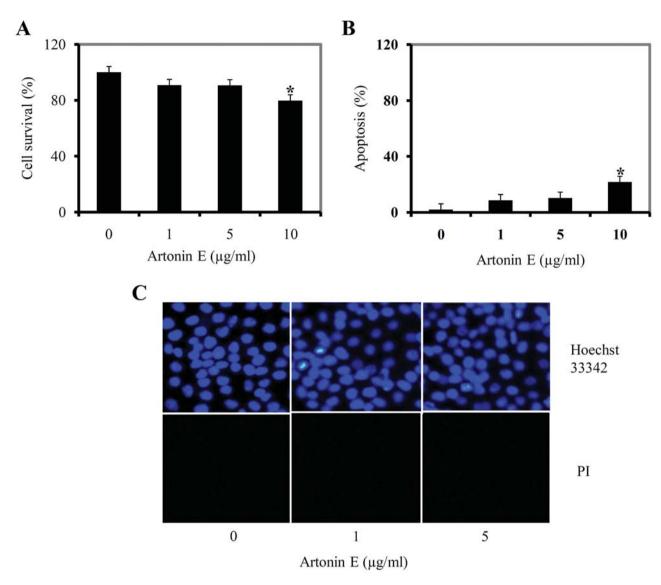
Figure 2. *Effect of artonin E on H460 lung carcinoma cell viability. Cells were treated with different concentrations of AE (0-10 μg/ml) for 24 h. A: Cell viability was determined by the 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay. Values are means±S.D. of triplicate experiments. B: Percentage of cell apoptosis was obtained from Hoechst 33342/ propidium iodide (PI) assays. C: Nuclear morphology of Hoechst33342/PI-stained cells was captured under fluorescence microscopy.*

Effects of AE on cell viability of HK2 normal human renal cells. Since a major concern for anticancer drug use is the cytotoxicity on normal cells, we tested whether AE at the mentioned concentrations caused significant toxicity to HK2 human renal cells. The renal cells were treated with AE at the concentrations of 0-5 μg/ml and cell viability, apoptosis, and necrosis were evaluated after 24 h. Figure 3A demonstrates that viability of AE-treated HK2 cells was not significantly altered in comparison to that of the non-treated controls. We also found a very limited number of apoptotic and necrotic cells in response to 1-5 μg/ml of AE. These results suggest

that AE at the concentrations of 1-5 μg/ml exhibited no cytotoxic effects towards neoplastic nor towards normal cells. *AE sensitizes H460 lung carcinoma cells to detachment-induced cell death*. Having shown that concentrations of AE of 0-5 μg/ml were non-cytotoxic to H460 lung cancer as well as HK2 normal renal cells, we further evaluated an anoikis-sensitizing effect of AE. H460 cells were detached and suspended in the presence or absence of AE at subtoxic concentrations and cell viability, apoptosis, and necrosis were analyzed at various times (0-24 h). Figure 4A shows that after detachment, H460 cells exhibited a gradual
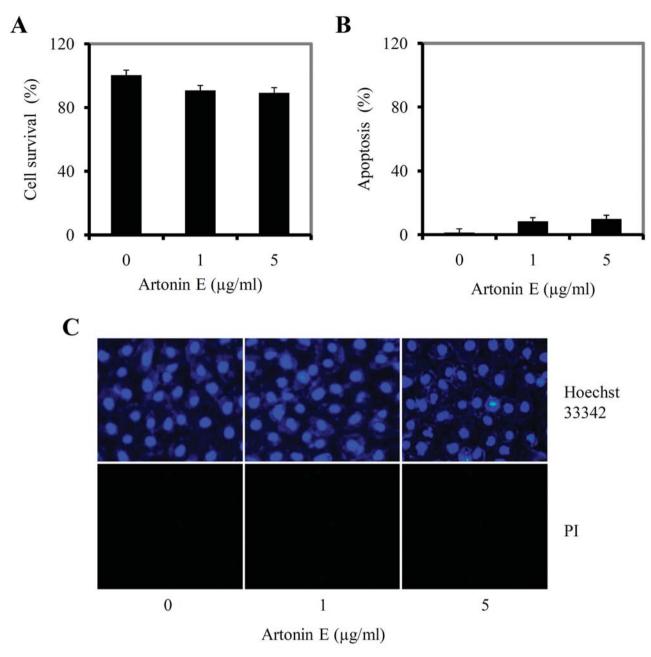
Figure 3. *Cytotoxic effect of artonin E in HK2 normal kidney cells. Cells were incubated in the presence or absence of AE (0-5 μg/ml) for 24 h. A: Cell viability was determined by the 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay. Values are means±S.D. of triplicate experiments. B: Percentage of cell apoptosis. C: Nuclear morphology of cells stained with Hoechst33342 and propidium iodide (PI) was visualized under fluorescence microscopy.*

decrease in cell viability over time. Importantly, treatment with AE significantly sensitized these cells to anoikis in a concentration-dependent manner. A significant decrease in cell viability, in comparison to that of non-treated controls, was observed as early as 6 h after cell detachment, with approximately 50% and 20% of cells remaining viable in response to 1 and 5 μg/ml of AE, respectively. In control

cells, approximately 85% cell viability was observed at 6 h after detachment and a dramatic decrease in cell viability was observed, firstly, at 24 h. To investigate the mechanisms of cell death, detached cells were incubated with Hoechst 33342 and PI fluorescent dyes and visualized and quantified under fluorescence microscopy. Figure 4B and C show a remarkable increase of the intensity of nuclear fluorescence
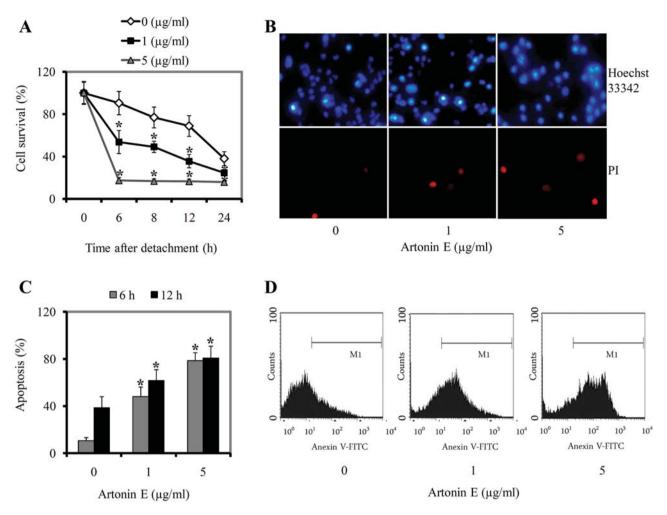
Figure 4. *Artonin E sensitizes H460 cells to detachment-induced apoptosis. A: Cells were treated with different concentrations of AE (0-5 μg/ml) and cell viability was determined by 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay at the indicated times. Values are means±S.D. of triplicate experiments. *p<0.05 versus non-treated control. B: Apoptosis and necrosis were detected by Hoechst 33342 and propidium iodide (PI) staining assay. C: Nuclear morphology of cells in response to AE treatment by staining with Hoechst 33342 and PI. D: Apoptosis was evaluated by annexin V-fluorescein isothiocyanate (FITC) and flow cytometry.*

and chromatin condensation of apoptotic cells. Approximately 70% and 80% apoptosis was recorded in response to 1 and 5 μg/ml AE treatment for 12 h, respectively, whereas necrotic cell death was minimal. Annexin-V staining assay was also performed to confirm the apoptotic response of the cells. Consistent with the above findings, annexin-V-stained cells detected by flow cytometry were found to increase in a dose-dependent manner in response to AE treatment (Figure 4D). These results indicated a potential role of AE in anoikis sensitization in human lung cancer cells.

MCL1 down-regulation is responsible for anoikis sensitization by AE. The mitochondrial apoptotic pathway has been shown to be involved in the process of cell anoikis (5-7). Among

anti-apoptotic members of BCL2 family proteins, MCL1 has garnered most attention since it has been shown to mediate anoikis resistance in many cancer cell types (18, 28, 29). The role of MCL1 on anoikis regulation was confirmed in the present study by stable plasmid transfection. Cells were transfected with MCL1-overexpressing, MCL1 knock-down, or control plasmids. After selection periods, transfectant cells were evaluated for their MCL1 levels by western blot analysis. Figure 5A shows that the highest MCL1 expression was detected in MCL1-overexpressing H460 (HMCL1) cells, while the lowest MCL1 expression was observed in short hairpin ribonucleic acid (shRNA) MCL1-transfected H460 (shMCL1) cells. Notably, control transfectants exhibited MCL1 levels comparable to those of the parental cells. These transfectants were evaluated for anoikis at different times. The
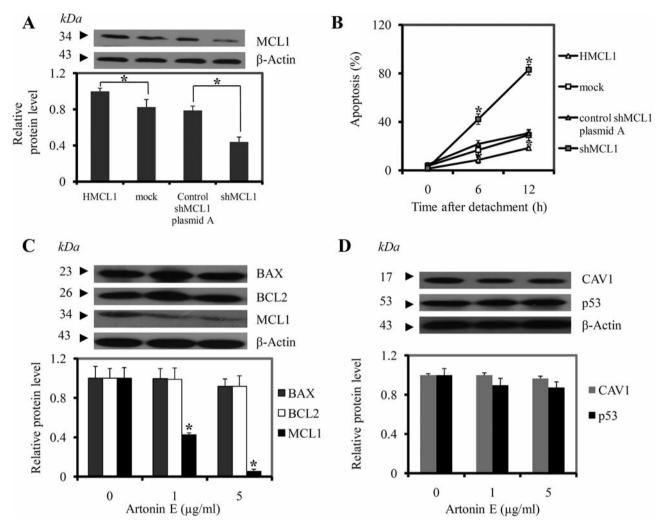
Figure 5. *Artonin E sensitizes anoikis via myeloid leukemia cell sequence-1 (MCL1) down-regulation. A: MCL1-mediated anoikis resistance in H460 cells. High expression of MCL1 protein (HMCL1), mock, control plasmid A and short hairpin MCL1 (shMCL1) cells were cultured in poly-2-hydroxyethyl methacrylate (HEMA)-coated plates and MCL1 levels were determined by western blotting at 12 h. B: Detached MCL1-overexpresing, -knock-down, and control cells were detached at 6, 12 h and viability of the cells was determined with the 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay. Values are means±S.D. of triplicate experiments. *p<0.05 versus mock-transfected control. Cells were detached and incubated with 1 and 5 μg/ml of AE for 12 h. C: B-cell lymphoma-2 (BCL2), BCL2-associated X protein (BAX) and myeloid leukemia cell sequence 1 (MCL1) expressions were determined by western blotting. D: p53 and caveolin-1 (CAV1) expressions were determined by western blot analysis. Values are means±S.D. (n=3). *p<0.05 versus non-treated control. Blots quantified related to β-actin, used to confirm equal loading of the samples.*

results showed that cell viability after detachment declined in a time-dependent manner in all cells. Corresponding to MCL1 levels in these cells, HMCL1 cells exhibited characteristics indicating the most anoikis resistance, with fewer than 10% of apoptotic cells being detected at 12 h after detachment. On the other hand, shMCL1 cells expressing the lowest level of MCL1 protein were shown to be very sensitive to detachment-induced apoptosis (Figure 5B). These results suggested that MCL1 plays an important role in anoikis regulation.

In order to clarify the mechanisms of AE in sensitizing cells to anoikis, the effect of AE on apoptosis-regulating proteins, namely p53, MCL1, BCL2, and BAX was evaluated. Detached H460 cells were treated with 1 and 5 μg/ml of AE or left untreated for 12 h and the expression of these proteins was evaluated by western blot analysis. Figure 5C and D show that while p53, BCL2 and BAX protein expressions were barely altered, the MCL1 expression significantly decreased in response to AE treatment (Figure 5C). Since the CAV1 protein has been shown in many
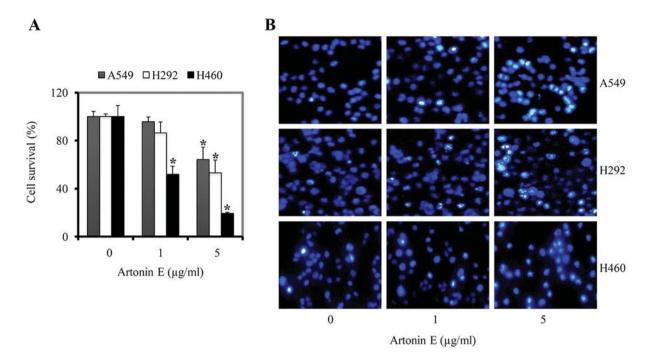
Figure 6. *Effect of artonin E (AE) of A549 and H292 lung cancer cells. A: A549, H292 and H460 cells were detached and cultured in poly-2-hydroxyethyl methacrylate (HEMA)-coated plates in the presence or absence of AE for 12 h and cell viability was determined as described in Materials and Methods. Values are means±S.D. (n=3). *p<0.05 versus non-treated control. B: Apoptotic and necrotic cells were detected by the Hoechst 33342 and propidium iodide (PI) staining assay. Representative photographs are shown from three independent experiments.*

studies to play an important role in anoikis resistance (16, 30-32), we also tested the effect of AE on CAV1 expression during anoikis. The results indicated that although CAV1 protein was found to be down-regulated after cell detachment in these cells (data not shown), AE treatment did not cause further reduction of expression of the protein (Figure 5D). These findings suggested that AE may, at least in part, have sensitized H460 cell anoikis *via* MCL1 down-regulation.

Anoikis-sensitizing effect of AE in A549 and H292 cells. Having demonstrated anoikis-enhancing activity of AE in H460 human lung cancer cells, we further confirmed such an effect in other lung cancer cell models. Anoikis after detachment of A549 and H292 human lung cancer cells in the presence or absence of AE was similarly evaluated, as mentioned above. Figure 6A shows that AE significantly reduced cell viability after detachment of both A549 and H292 cells. Even though A549 and H292 cells exhibited less susceptibility to AE sensitization, both cells exhibited reduced viability in response to 5 μg/ml of AE, with approximately 70% of A549 and 50% of H292 cells remaining viable in comparison to 10% of H460 cells under the same conditions. Apoptotic cells were also remarkably increased in these cells in response to AE treatment, correlating with earlier results (Figure 6B). These results

indicated the general role of AE in anoikis sensitization of lung cancer cells.

Discussion

Anoikis resistance enables cancer cells to spread and establish their secondary tumors (5-7). In lung cancer, metastasis is considered as the most important cause of death (33-35) and such a concept has lead to the development of novel anti-metastasis agents and strategies (36-40). Considering plants as an important source of pharmacologically active compounds, AE, a compound isolated from the bark of *Artocarpus gomezianus* was shown to possess several activities such as arachidonate 5-lipoxigenase inhibition, anti-microbial, anti-malarial and anti-tuberculosis activity, and cytotoxicity (24-27). AE was also demonstrated to have a cytotoxic effect against p-388 leukemia cells (26). However, there is no evidence indicating the effect of this compound on cancer metastasis. We have reported, to our knowledge for the first time, that AE at concentrations with minimal toxic effects on normal renal cells exhibited a significant anoikis-sensitizing activity against lung cancer cells. We found that AE, at 5 μg/ml, significantly sensitized H460, A549, and H292 lung cancer cells to detachment-induced apoptosis and provided evidence of MCL1 being involved in the underlying mechanism.

Anoikis is a form of apoptosis initiated by loss of or inappropriate contact with the extracellular matrix or surrounding cells (5-7). Cancer cells either acquire anoikis resistance or possess an innate-resistant ability which enables them to survive after detachment, travel in blood or lymphatic circulations, and establish themselves at distant locations (7, 41, 42). Mechanisms responsible for anoikis resistance in cancer have been intensively investigated and the key proteins, which are MCL1 and CAV1 have garnered dominant attention. Overexpression of CAV1, a major protein component in caveolae, has been shown to mediate anoikis resistance in lung cancer cells (31, 32). Evidence was further provided that expression of such a protein is related to poor prognosis in lung cancer (43, 44). Likewise, MCL1 was shown to mediate anoikis resistance in melanoma (18), as well as lung cancer cells (29). As MCL1 is classified as an antiapoptotic protein, it functions in interacting and neutralizing pro-apoptotic functions of pro-apoptotic proteins, preventing the release of cytochrome *c* from mitochondria (45, 46). Herein, we found that AE reduced the cellular levels of MCL1 in detached cells. The results in this study showed that MCL1 down-regulation mediated by shRNA also resulted in increasing sensitivity of the cells to anoikis (Figure 5B). These results, together with our finding that other proteins, such as BCL2, BAX, CAV1, and p53, were not affected by the addition of AE indicates that AE may sensitize cells to anoikis by reducing cellular MCL1 expression. Since MCL1 was shown to be an important therapeutic target for the treatment of many types of cancer (47-49), compounds targeting MCL1 protein, such as AE, could be of greater interest for development and use in cancer therapy.

In summary, the present study has provided information regarding the role of AE in the regulation of anoikis in lung cancer cells. Detailed molecular analysis of AE on anoikis-resistant cells provides insights into the mechanisms of this compound which may be useful for the development of novel therapeutic strategies for prevention of cancer dissemination.

Acknowledgements

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