Lupalbigenin from *Derris scandens* Sensitizes Detachment-induced Cell Death in Human Lung Cancer Cells

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Abstract. *Background/Aim: The ability of cancer cells to resist to anoikis has been shown to augment cancer cell metastasis in many cancers. In search for potential substances for anti-metastatic approaches, this study aimed to investigate anoikis-sensitizing activity of lupalbigenin, extracted from Derris scandens. Materials and Methods: Human lung cancer cells were treated with non-cytotoxic concentrations of lupalbigenin in a detachment condition. Anoikis was evaluated at various time points using MTT viability assays. The effect of lupalbigenin on anchorage-independent growth was performed by soft-agar assay. The survival signaling proteins, as well as regulatory proteins of apoptosis and metastasis, were examined by western blot analysis. Results: Lupalbigenin treatment significantly down-regulated survival proteins, including protein kinase B (pAKT/AKT) and extracellular signal-regulated kinase (pERK/ERK), as well as anti-apoptotic protein B-cell lymphoma 2 (BCL-2), resulting in the enhancement of the cellular response to anoikis and the decrease of growth and survival in an anchorage-independent condition. Conclusion: Lupalbigenin sensitizes detachment-induced cell death in human lung cancer cell through down-regulation of pro-survival proteins.*

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Lung cancer has been recognized for its high mortality rate (1). Metastasis in lung cancer to secondary organs or tissues has been accepted as an important course of severe complications, as well as mortality (2-4). Unfortunately, lung cancer is frequently diagnosed with the presence of metastasis at the time of first detection, which leads to unsuccessful treatment of chemotherapeutic drugs and the recurrence of cancer pathology (5-8).

The capability of cancer cells to survive and proliferate in detached or anchorage-independent condition is a critical trait found in metastatic cancer cells. In order to spread, cancer cells detach from their original tumor, escape from anoikis, enter in circulation and establish secondary tumors (9-12). Recently, sufficient *in vivo* studies have demonstrated the promising therapeutic potential of anoikis-sensitizing agents in inhibition of tumor formation (13-15). The survival proteins, such as protein kinase B (AKT) and extracellular signal-regulated kinase (ERK), have been shown to play a critical role in attenuating anoikis response (16, 17). Moreover, the anti-apoptotic protein members of B-cell lymphoma 2 (BCL2) family proteins and metastasis-regulating Caveolin-1 (CAV-1) were demonstrated to have a crucial role in anoikis-resistant and metastatic cancer cells (18-22).

According to ongoing investigations in search of anti-metastatic compounds, natural product-derived compounds have been shown to have several promising effects (23-27). As the extracts from *Derris scandens*, a Thai medicinal plant, was shown to have anticancer activity (28, 29), the present study aimed to further reveal the anti-metastatic potentials of lupalbigenin, a pure compound extracted from *D. scandens*.

Materials and Methods

Cell culture. H460 human non-small lung cancer cells (American Type Culture Collection (ATCC), Manassas, VA, USA) were cultured in RPMI medium supplemented with 10% fetal bovine serum (FBS), 2 mmol/l L-glutamine and 100 units/ml penicillin/

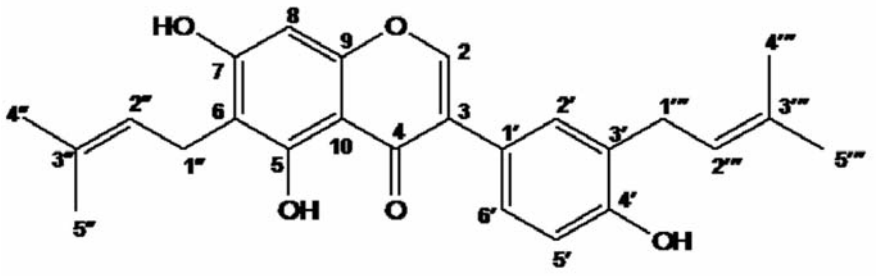

Figure 1. *Chemical structure of lupalbigenin ($C_{25}H_{26}O_5$), molecular weight 406.*

streptomycin (Gibco, Gaithersburg, MA, USA). Cells were maintained in a humidified incubator containing 5% CO_2 at 37˚C until reaching 70-80% confluence before experiments.

Chemical reagents and plant material. Lupalbigenin (Figure 1) (>98%) was isolated from stems of *Derris scandens*. The voucher specimen (SS-SPT006) was deposited at the Herbarium of Natural Medicine, Chulalongkorn University. Briefly, the dried stems (3 kg) were powdered and extracted with MeOH (12 l) at room temperature to yield a viscous mass (277.8 g) after evaporation of the solvent. The material was partitioned with EtOAc and water (4×300 ml) to give an EtOAC extract (87.9 g) and water extract (10.9 g), respectively. EtOAc was subjected to vacuum-liquid chromatography on silica gel (*n*-hexane-EtOAc, gradient) to give 7 fractions (F1-F7). Fraction F4 (4 g) was separated by CC over silica gel (n-hexane-EtOAc 6:4) to give lupalbigenin (48.5 mg). The structure of lupalbigenin was determined through analysis of its spectroscopic data. All chemical substance including Hoechst33342, 2,3-b-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide salt (XTT), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), dimethysulfoxide (DMSO) and agarose were obtained from Sigma Chemical, Inc. (St. Louis, MO, USA). Antibody of Erk, pErk (Thr202/Thr204), Akt, pAkt (Ser 473), Bcl-2, caveolin1, β-actin and specific horseradish peroxidase (HRP)-link secondary antibody were obtained from Cell Signaling Technology, Inc. (Danver, MA, USA).

Cell viability assay. MTT assay was used to evaluate cell viability. After treatment with 0-100 μM of lupalbigenin for 24 h, H460 cells were incubated with 0.4 mg/ml of MTT solution at 37˚C in a dark place for 4 h. Then, the formazan crystal was dissolved by replacement of MTT solution with 100 μl/well of DMSO. The intensity of formazan color was measured at 570 nm using a microplate reader, Anthros (Durham, NC, USA). The analyses were established in at least three independent replicate cultures. Cell viability was calculated from the optical density (OD) ratio of treated to non-treated control cells and presented as a percentage in relation to the non-treated controls.

Anoikis assay. A single-cell suspension of H460 cells in culture medium was seeded into an ultra-low attachment plate (Corning, Acton, MA, USA) at a density of 1.5×10^5 cells/ml. The cells were treated or left untreated with non-toxic concentration of lupalbigenin. After 0, 3, 6, 9, 12 and 24 h, the cells were harvested and incubated with 20 μM of XTT for detection of cell viability. After light protection at 37˚C for 4 h, the intensity of the formazan product from XTT was measured at 450 nm using a microplate reader.

Nuclear staining assay. Modes of cell death were evaluated under a fluorescent microscope (Olympus IX51 with DP70; Olympus, Melville, NY, USA). After treatment with lupalbigenin, H460 cells were co-stained with 10 μM of the Hoechst33342 dye and 5 μg/ml of the PI dye for 30 min at 37˚C in the dark. Apoptotic and necrotic cells were observed as staining of blue fluorescent Hoechst33342 and red fluorescent PI, respectively.

Anchorage-independent growth assay. The effect of lupalbigenin on cell growth in detachment condition was investigated in a two-layer of soft agar assay. Agarose solution at 0.5% w/v in RPMI medium was set as bottom layer (500 μl/well) in a 24 well-plate. Meanwhile, the single-cell suspensions of l lupalbigenin-treated H460 cells in 0.33% agarose (1,500 cells/250 μl) were prepared for the top layer. After the upper layer was set in at 37˚C for 4 h in the incubator, the culture medium was added on top. Fresh complete RPMI medium (250 μl/well) was added every 3 days. The colonies were allowed to form for 14 days and, then, number and size of colonies were evaluated under a microscope (Olympus IX51 with DP70).

Western blot analysis. Briefly, H460 cells, which had been treated with lupalbigenin or left untreated, were harvested and lysed on ice for 60 min. The BCA protein assay kit (Pierce, Rockford, IL, USA) was used to determined protein content in cell lysates. An equal amount of protein of each sample was separated by size using SDS-PAGE and, then, transferred to nitrocellulose membranes. Before incubation with specific primary antibodies, the membrane was blocked in 5% skim milk in TBST (25 mmol/l Tris-HCL, pH 7.4, 125 mmol/l NaCl, 0.1% Tween 20) for 1 h at room temperature. The probed membranes (4˚C for 12 h) were washed with TBST for 8 min three times. Then, the membrane was probed with HRP-conjugated secondary antibody for 2 h at room temperature. The signal of immunoreactive proteins was detected by enhanced chemiluminescence (Supersignal West Pico; Pierce, Rockford, IL, USA). The quantitative analysis was performed with the analyst/PC densitometry software (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis. Mean data from at least three independent experiments were normalized to result in the non-treated control. Statistical analysis was performed using one-way ANOVA. A *p*-value of less than 0.05 was considered statistically significant.

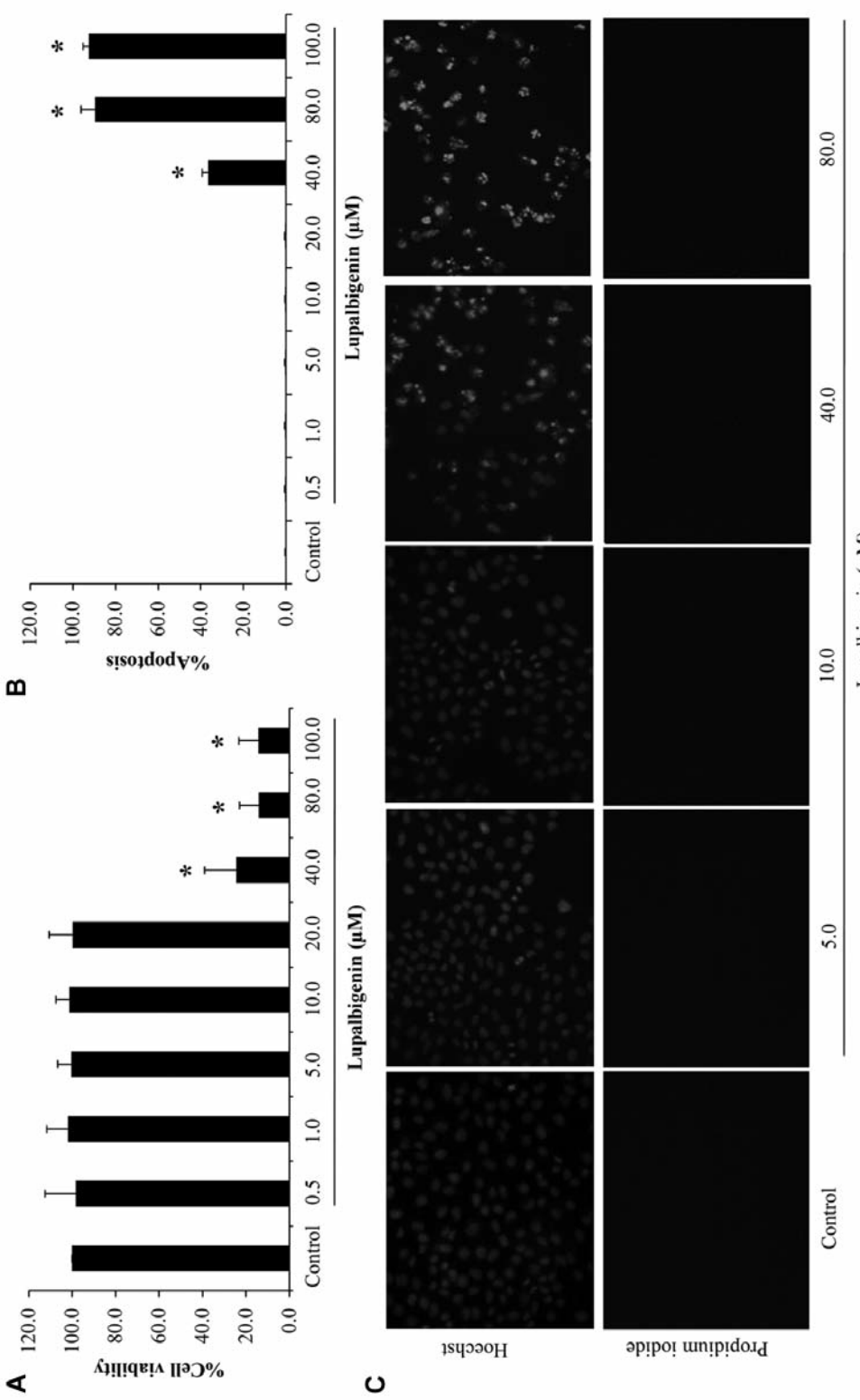

Figure 2. *Effect of lupalbigenin on cell viability. (A) H460 cells were treated with various concentrations of lupalbigenin (0-100 μM) for 24 h and, then, viable cells were examined by MTT assay. (B) Apoptosis in lupalbigenin-treated H460 cells was compared to non-treated control cells. Data represent the means±SD (n=3). *p<0.05 versus untreated control cells. (C) Apoptotic and necrotic cell death was evaluated using Hoechst33342/PI co-staining using the same as above (A) treatment protocol.*

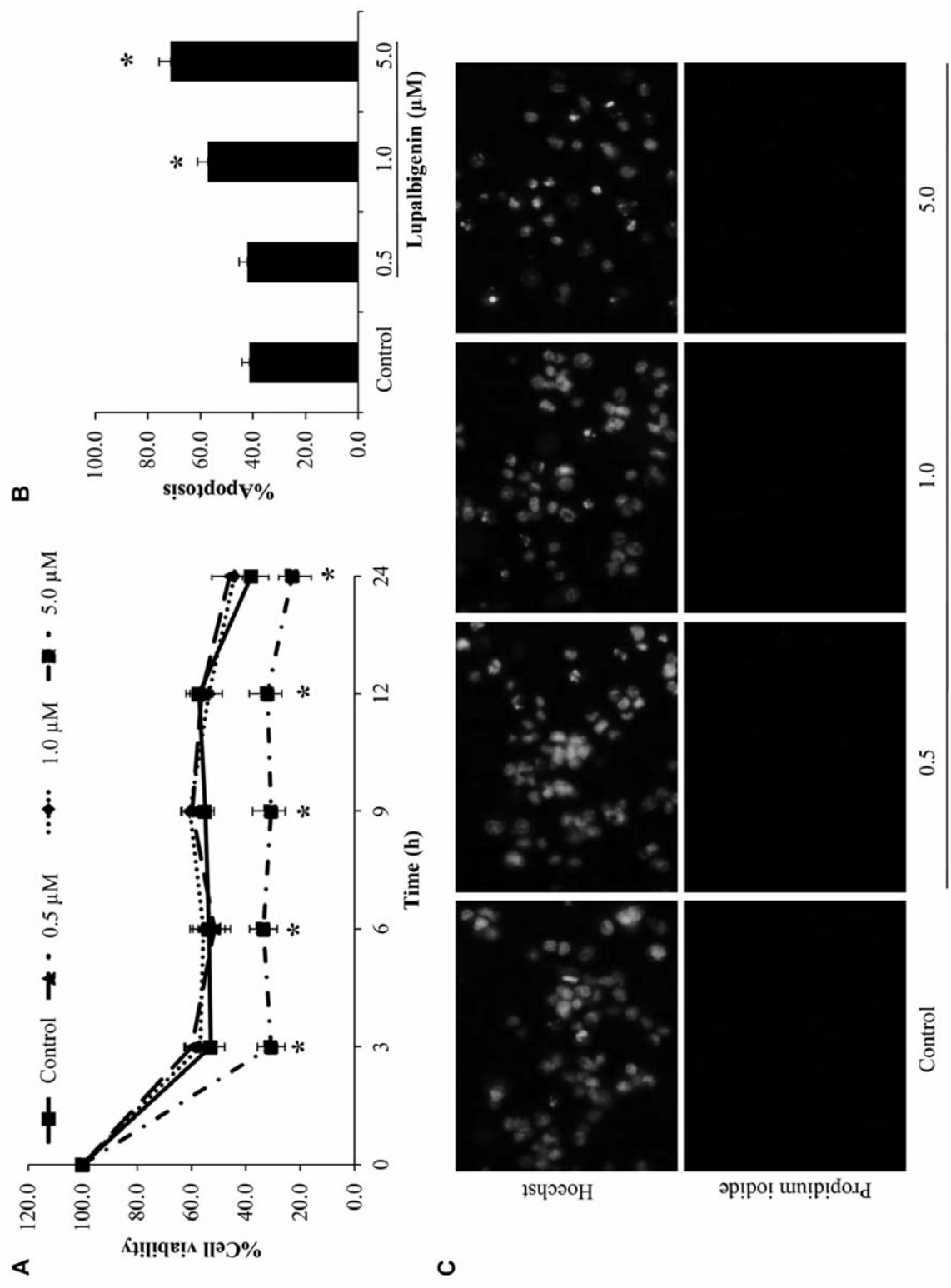

Figure 3. *Effect of lupalbigenin on detachment-induced cell death. (A) H460 cells in detachment condition were treated with various non-toxic concentrations of lupalbigenin (0-5 μM). At the indicated time points, cell viability was examined by the XTT assay. (B) %apoptosis in detachment condition was analyzed via bright fluorescence of Hoechst33342 staining. Data represent the means±SD (n=3). * p<0.05 versus control cells at each time point. (C) Morphology of anoikis nuclei stained with Hoechst33342 dye and propidium iodide.*

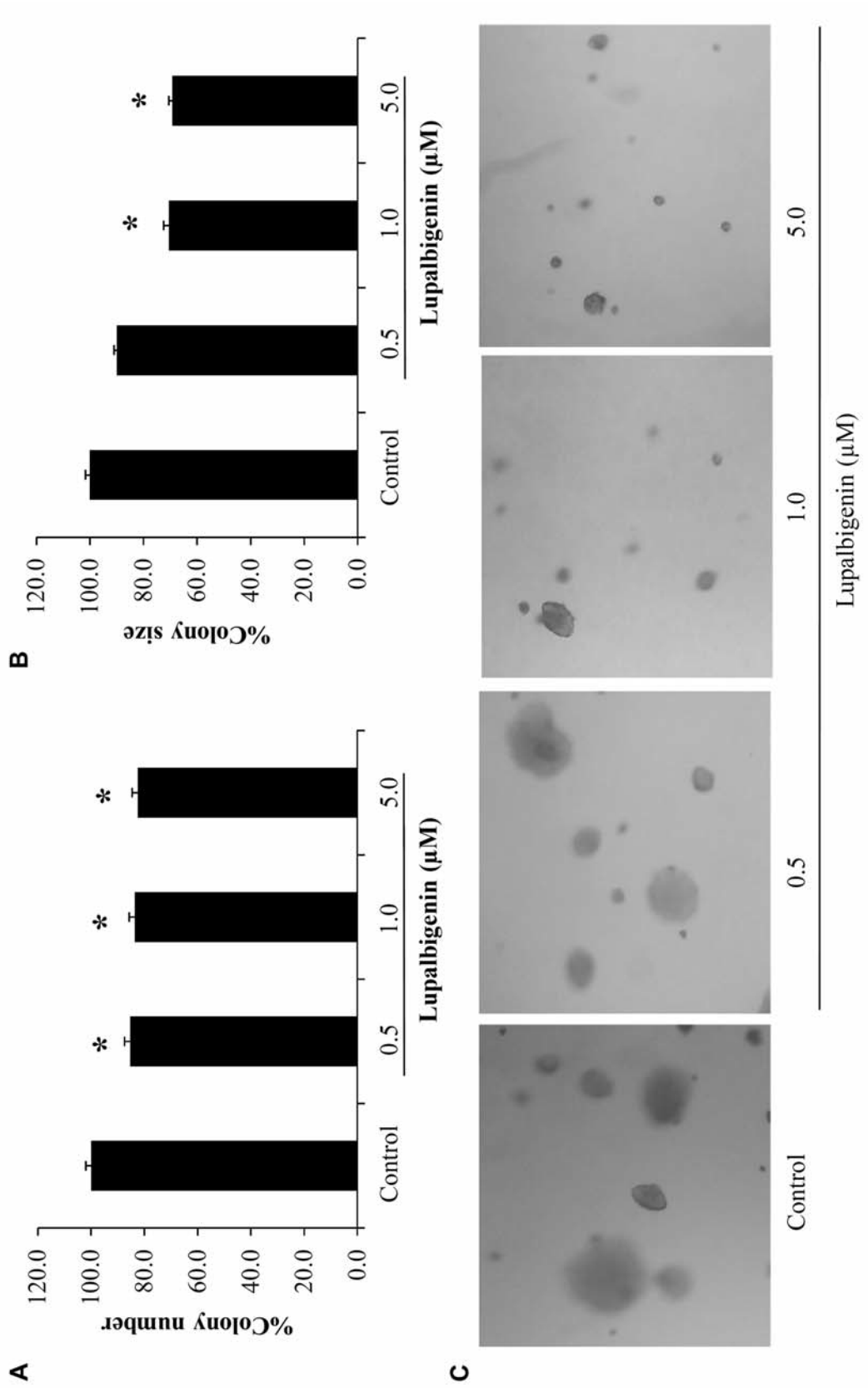

Figure 4. *Effect of lupalbigenin on anchorage-independent growth. Lupalbigenin-treated H460 cells were subjected to anchorage-independent growth assay for 2 weeks. (A) Colony number and (B) size were counted and presented relative to the untreated control cells. Data represent the means±SD (n=3). *p<0.05 versus untreated control cells. (C) Colonies were visualized under a microscope (magnification ×4).*

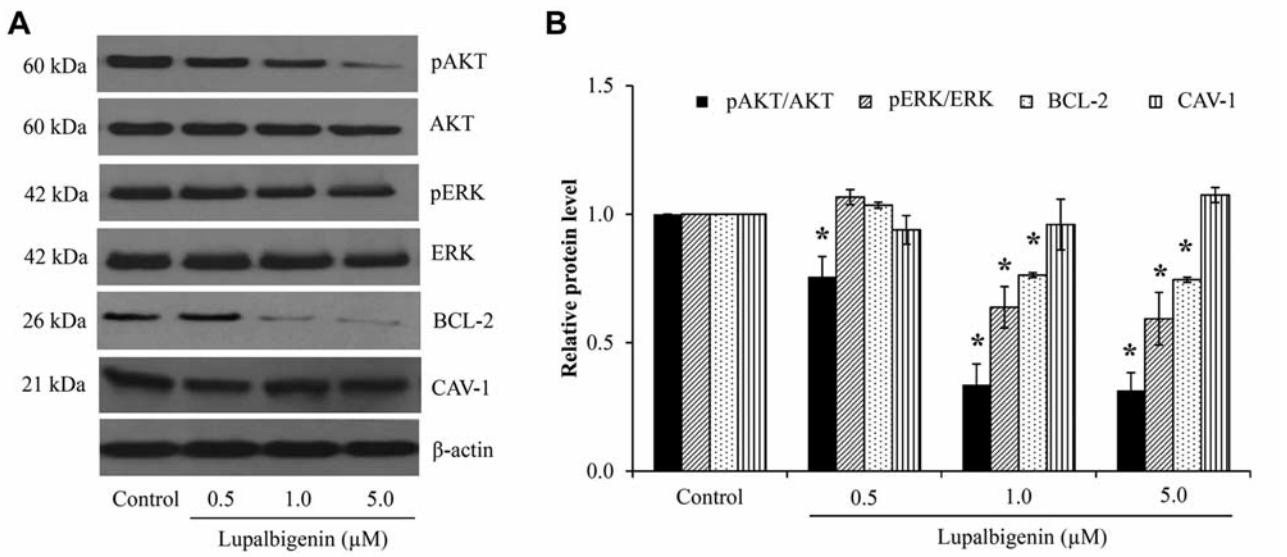

Figure 5. *Effect of lupalbigenin on survival pathway and anti-apoptotic proteins. (A) H460 cells were treated under various concentrations of TDB (0-5 μM) for 24 h collected and analyzed for protein kinase B (AKT), phosphorylated AKT (Ser473; pAKT), extracellular signal-regulated kinase (ERK) and phosphorylated ERK (Thr202/Thr204; pErk) expressions by western blot analysis. Additionally, anti-apoptotic BCL-2 (B-cell lymphoma 2) protein and Caveolin-1 (CAV-1), a metastatic regulating protein, were also investigated in lupalbigenin-treated cells. (B) The immunoblot signals were quantified by densitometry and mean data from three independent experiments were presented. Data represent the means±SD (n=3). *p<0.05 versus untreated control cells.*

Results

Evaluation of the cytotoxic effect of lupalbigenin on H460 cells. The cytotoxic effect of the compound on human lung cancer was first characterized. Cells were treated with various concentrations of lupalbigenin (0-100 μM) for 24 h and viability of the cells was determined. Figure 2A shows that lupalbigenin at a concentration greater than 20 μM significantly decreased cell viability of H460 cells. The apoptotic evaluation further confirmed the cell viability results; treatment of the cells with 40-100 μM lupalbigenin significantly increased cell apoptosis (Figure 2B). The effect of lupalbigenin on apoptosis of cells was evaluated by Hoechst33342/PI co-staining assay (Figure 2C). The nuclear morphology analysis, together with the MTT cell viability assay, indicated that lupalbigenin at concentrations of 0-20 μM caused no significant cytotoxic effect as indicated by the comparable viability to the non-treated control and the absence of condensed and/or fragmented nuclei (Figures 2A-C).

Lupalbigenin sensitizes anoikis in H460 cells. In order to investigate the anoikis-sensitization activity of lupalbigenin, cells were suspended in culture media containing a non-toxic concentrations of lupalbigenin (0-5 μM) in non-adhesive poly-HEMA coated-plates for 0-24 h and cell survival was determined by the XTT assay. Figure 3A indicates that cell survival was gradually decreased over time in the non-treated control group. Interestingly, treatment of the cells with 5 μM lupalbigenin significantly reduced cell survival in detached condition. The significant decrease of cell survival was detectable as early as 3 h after treatment with the compound. Furthermore, apoptosis and necrosis evaluation revealed that lupalbigenin significantly increased the anoikis response of the cells without necrosis-inducing effect as indicated by the absence of PI-positive cells (Figure 3C). Taken together, these results demonstrated herein that lupalbigenin at non-toxic concentrations significantly sensitized the lung cancer cells to anoikis.

Lupalbigenin attenuates growth and survival in anchorage-independent condition. As the growth and survival of cancer cells in detached condition have been accepted to be critical prerequisite capabilities of success of metastatic cancer cells, we next investigated whether lupalbigenin could inhibit such capabilities in H460 lung cancer cells. For this, cells were cultured in the absence or presence of lupalbigenin in an anchorage-independent soft-agar growth assay for 2 weeks and the number and size of cell colonies were determined, as described. Figure 4A shows that treatment of the cells with 0.5-5 μM of the compound under investigation significantly reduced the colony number in comparison to that of non-treated control cells. Moreover, lupalbigenin, at concentrations of 1 and 5 μM, was shown to significantly reduce the colony size in such an assay (Figure 4B). Taken

together, our results indicated the potential of lupalbigenin in inhibition of tumor cell growth and survival in anchorage-independent condition.

Lupalbigenin decreases anti-apoptotic and survival proteins. Having shown that lupalbigenin possesses anoikis sensitization, as well as inhibition of tumor growth in detached conditions, we next investigated the underlying mechanisms. To clarify the mechanisms of the lupalbigenin in regulating anoikis, we thus determined the effects of the compound on proteins regulating anoikis of the cells, namely activated AKT (pAKT), activated ERK (pERK), BCL-2 and CAV-1. Western blot analysis revealed that treatment of the cells with lupalbigenin significantly down-regulated the levels of activated AKT and ERK in comparison to those of non-treated control. We also found that the compound decreased the cellular level of anti-apoptotic BCL-2 proteins. It is worth noting that the patterns of CAV-1 protein were only slightly affected by the lupalbigenin treatment (Figures 5A, B).

Discussion

Metastasis of cancer cells is long-known as an important cause of cancer-related death. Several critical abilities of cancer cells for successful spreading have been indentified and garnered increased attention in the anti-cancer research field. Among multiple factors facilitating the potentiality of cancer cells to spread, anoikis resistance and growth, in an anchorage-independent condition, have been shown to be hallmarks of aggressive cancer characteristics (30, 31). To metastasize, detached cancer cells must increase their survival signals to survive. Indeed, the dissociation of cells in the extracellular matrix during cell detachment will cause a dramatic reduction of cellular survival signals, including AKT and EKR, resulting in anoikis of the cells. A number of metastatic cancer cells have been reported to have an augmented level of activated AKT and activated ERK, signals that confer resistance to anoikis (17, 32, 33).

Furthermore, evidence indicate that the increase of the anti-apoptotic protein members of the BCL-2 family can inhibit the apoptotic process induced by the cellular detachment (20, 34, 35). Therefore, compounds that have the potential to suppress such survival or anti-apoptotic proteins in metastatic cancer cells are recently of interest for anti-metastatic approaches.

Herein, we demonstrated -for the first time- that lupalbigenin, a compound isolated from *Derris scandens*, has the potential to be considered for anti-metastatic therapeutics. Lupalbigenin significantly increased the anoikis response of the lung cancer H460 cells (Figure 2) and could also inhibit tumor growth, as well as survival in an anchorage-independent condition (Figure 4). Our study demonstrated that lupalbigenin treatment resulted in a significant down-regulation of anti-apoptotic proteins like BCL-2 and the survival-related proteins, namely AKT and ERK. These results, taken together with previous finding of the anticancer activity of the extract form the same plant (28, 29), demonstrated the potentiality of this compound to be further developed for the treatment of lung cancer.

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