Abstract. Anthricin (deoxypodophyllotoxin) is a major lignan in Anthriscus sylvestris and possesses many bioactivities such as antiproliferative, antitumor, anti-platelet aggregation, antiviral and anti-inflammatory actions. However, the anticancer effects of anthricin on A549 human non-small cell lung cancer cells and potential molecular mechanisms remain unknown. Therefore, we investigated the anticancer effect of anthricin and the underlying mechanism in A549 cells. Anthricin (10‑200 nM) inhibited the viability of A549 cells in a dose- and time-dependent manner. Moreover, anthricin-induced apoptosis was confirmed by live and dead assay, 4,6-diamidino-2-phenylindole staining, and flow cytometric analysis. In addition, anthricin induced cell cycle arrest at the G2/M phase through suppression of the expression of cell cycle cascade proteins, Cdc2 and Cdc25C. Furthermore, it induced the expression of caspase-related proteins and significantly suppressed the phosphorylation of insulin-like growth factor 1 receptor (IGF1R), PI3K and Akt. Anthricin significantly inhibited tumor growth without any significant change in the body weight of mice in A549 tumor xenograft BALB/c nude mice. Anthricin induced caspase-dependent apoptosis through the IGF1R/PI3K/Akt signaling pathway in A549 cells.

Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide, with a 5-year survival rate of ~15% (1,2). Based on cell size, lung cancer is classified into small cell lung cancer (SCLS), which accounts for 15-20% of all lung cancers, and non-small cell lung cancer (NSCLC), which accounts for the remaining ~80%. Adenocarcinoma accounts for 40% of NSCLC, and the prognosis is poor (3,4). Unfortunately, the drugs currently used against various kinases, such as mutant EGFR, are ineffective in patients with lung cancer owing to variable, transient, and incomplete responses (5). Thus, novel therapies are an unmet need for lung cancer patients, in order to improve the progression of the disease.

Anthriscus sylvestris (L.) Hoffm. belongs to the Apiaceae (syn. Umbelliferae) family and grows in hedges, road verges, and neglected pastures in Europe, North America, Asia, and New Zealand (6). In Asia and China, the dried roots of A. sylvestris are used in traditional medicine for its antipyretic and analgesic properties and as cough remedy and the young aerial parts are used as food (7). A. sylvestris has a high content of lignans. In several studies, monoterpenes, anthricinol, deoxypodophyllotoxin, and angeloylbutenoic acid were separated by hexane-soluble fraction and anthricin, isoanthricin, and crocactone were isolated by EtOAc-soluble fraction (8,9). Anthricin (deoxypodophyllotoxin) is a major lignan in this plant and has many bioactivities such as antiproliferative, anti-tumor, anti-platelet aggregation, antiviral, anti-inflammatory and liver protective actions (8,10-12). Numerous studies have reported the anticancer effect of anthricin on various cancer cells, such as gastric and breast cancer, cervical carcinoma and lung cancer, through G2/M cell cycle arrest, microtubule formation inhibition, and caspase-dependent apoptosis (13,14). Jung et al reported that anthricin isolated from A. sylvestris suppresses the growth of breast cancer cells by inhibiting Akt/mTOR signaling and enhancing autophagy inhibition (15). However, the mechanism underlying this biological phenomenon remains unknown.

Insulin-like growth factor 1 receptor (IGF1R) is a transmembrane receptor tyrosine kinase receptor and highly expressed in various cancers such as lung adenocarcinoma, pancreatic carcinoma, and breast cancer (16). IGF1R signaling on the cell membrane mediated by IGF-1 has a vital role in cell proliferation and differentiation as well as metabolism and against apoptosis (17). When IGF-1 binds to the α domain of IGF1R, the β domain of IGF1R is activated by auto-phosphorylation on specific tyrosine residues and

Key words: anthricin, A549 human non-small cell lung cancer cells, IGF1R/PI3K/Akt signaling pathway, cell cycle arrest, apoptosis
switches on the downstream signaling, such as the PI3K/AKT pathway and the RAS/RAF/MEK pathway (18,19). Recently, the clinical significance of IGF1R expression in human NSCLC was reported, and the results revealed that high IGF1R expression on the membranes was predictive of poor progression-free survival (PFS) in adenocarcinoma (20). These findings indicated that IGF1R could be a potential therapeutic target. Thus, we hypothesized that anthricin regulates cell apoptosis through IGF1R/PI3K/AKT signaling. In the present study, we evaluated the effects and mechanism of action of anthricin isolated from *A. sylvestris* in A549 human NSCLC cells.

**Materials and methods**

*Preparation of Anthricin from A. sylvestris (L.) Hoffm.*

Dried *A. sylvestris* roots were extracted with 10 volumes of methanol (v/w) and the sublayer organic phase was concentrated in a rotary vacuum evaporator (Eyela, Tokyo, Japan) and lyophilized. The residue was dissolved in dimethyl sulfoxide (DMSO), filtered, and analyzed using a Shimadzu HPLC system (Shimadzu Corporation, Kyoto, Japan) consisting of an LC-20AR pump, an SCL 10A system controller and an SPD-20A UV-VIS detector. Semi-preparative HPLC for purification of the methanol extract of dried *A. sylvestris* roots: preparative reversed-phase HPLC was performed using a Shimpack PRP-ODS column (250x20 mm I.D., 5 µm; Shimadzu). The mobile phase was a mixture of two liquids distributed by (A) water and (B) acetonitrile at a flow rate of 1.0 ml/min. The elution program commenced at 95% A: 5% B followed by a linear gradient for 60 min to 5% A: 95% B. The sample injection volume was 1 ml, and the detection by UV was set at a wavelength of 210 nm. Chemical identification was performed by comparing the retention times and mass spectra of the targeted peaks with those of the standard sample. Anthricin (standard sample; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was dissolved in DMSO at 10 mg/ml. Anthricin isolated from *A. sylvestris* roots prepared from the methanol extract of dried *A. sylvestris* roots was dissolved in DMSO at 10 mg/ml.

**Cell culture.** A549 human NSCLC cells were purchased from the Korean Cell Line Bank (Seoul, Korea). Cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 100 U penicillin-streptomycin at 37°C in a 5% CO₂-humidified incubator.

**Cell viability assay.** The cytotoxicity of anthricin was assessed using the MTT assay. In brief, the cells were seeded into a 12-well plate (5x10^5 cells/ml) and allowed to adhere overnight. The cells were then treated with either vehicle DMSO or anthricin (0, 10, 20, 50 and 200 nM) for 24 and 48 h. Following incubation, 100 μl of MTT solution (5 mg/ml) was added to each well and the plate was incubated for 4 h at 37°C. The resulting formazan crystals were dissolved in DMSO, and the optical density (OD) was assessed at 570 nm using a microplate reader (Bio-Tek Instruments, Winooski, VT, USA). The cell viability rate was calculated using the following equation: means of OD<sub>mean</sub>/means of OD<sub>control</sub> x 100%.

**Live and dead assay and DAPI staining of cells.** A549 cells were seeded in a 4-well chamber slide a at a density of 1x10^3 cells/well. After overnight incubation, the cells were treated with different concentrations of anthricin (0, 10, 20 and 50 nM) for 24 h. Flow cytometric analysis. A549 cells were treated with anthricin at concentrations of 0, 10, 20 and 50 nM for 24 h, harvested, and washed with ice-cold PBS twice. For the apoptosis analysis, the cells were stained using a PE-Annexin V/7-AAD apoptosis detection kit (BD Biosciences) according to the manufacturer's instructions. For DAPI staining, the treated cells were washed, fixed with 4% formaldehyde in PBS at room temperature for 10 min, and stained with 300 nM DAPI for 20 min at room temperature. The stained cells were washed thrice with PBS and observed under a fluorescence microscope (Eclipse TE2000; Nikon Instruments, Melville, NY, USA). Cells exhibiting condensed and fragmented nuclei were considered to have undergone apoptosis.

**Flowcytometricanalysis.** A549 cells were treated with anthricin at concentrations of 0, 10, 20 and 50 nM for 24 h, harvested, and washed with ice-cold PBS twice. For the apoptosis analysis, the cells were stained using a PE-Annexin V/7-AAD apoptosis detection kit (BD Biosciences) according to the manufacturer's protocol and subsequently analyzed by flow cytometry (BD FACSCalibur). The quantitative data was expressed as density plots using WDI software. Non-stained cells (Annexin V and 7-AAD negative) were considered viable, cells stained with Annexin V positive and 7-AAD negative were regarded as early apoptotic cells, and cells stained with both Annexin V and 7-AAD were considered late apoptotic or already dead cells. For cell cycle analysis, after cells were treated with anthricin for 24 h, as previously described, they were harvested and washed in ice-cold PBS. The cell pellets were fixed with 4% formaldehyde in PBS for 15 min and stained with 50 μg/ml propidium iodide and 100 μg/ml RNase for 20 min. The data were analyzed using Attune NxT Acoustic Focusing Cytometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

**Western blot analysis.** A549 cells were treated with anthricin at concentrations of 0, 10, 20 and 50 nM for 24 h. The cells were lysed with protein extraction reagent (iNtRON Biotechnology, Sungnam, Korea) for 30 min on ice. The supernatant was transferred to a new tube after centrifugation at 13,000 x g for 15 min at 4°C (Sorvall Centrifuge, Bad Homburg, Germany). Then, the protein concentrations were quantified using the BCA protein assay (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with bovine serum albumin (BSA) as a standard.
Equal amounts of protein (20 μg/lane) were separated by 8 or 15% SDS-PAGE gels under reducing conditions, followed by electrophoretic transfer onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked with 5% BSA for 1 h. The membranes were incubated at 4°C overnight with the following primary antibodies diluted to 1:1,000: Bax (cat. no. 2772), Bcl-2 (cat. no. 2872), cleaved caspase-8 (cat. no. 9496), cleaved caspase-3 (cat. no. 9661), cleaved caspase-9 (cat. no. 7237), PARP (cat. no. 9542), Cdc2 (cat. no. 28439), Cdc25C (cat. no. 4688), p-IGF1R (Y1131) (cat. no. 9750), IGF1R (cat. no. 3021), PI3K (cat. no. 4292), p-PI3K (cat. no. 4228), AKT (cat. no. 9272), p-AKT (cat. no. 9271), mTOR (cat. no. 2971) (all from Cell Signaling Technology, Beverly, MA, USA) and β-actin (1:5,000; cat. no. YIF-LF-PA0207A; AB Frontier, Seoul, Korea). After washing with Tris-buffered saline and Tween-20 (TBST) thrice, the membranes were incubated for 1 h at room temperature with HRP-labeled secondary antibodies, anti-rabbit IgG (cat. no. 7074) and anti-mouse IgG (cat. no. 7076) diluted to 1:5,000 from Cell Signaling Technology. Western blotting was performed to evaluate whether A549 cell membranes were incubated at 4°C overnight with the following primary antibodies diluted to 1:1,000: Bax (cat. no. 2772), Bcl-2 (cat. no. 2872), cleaved caspase-8 (cat. no. 9496), cleaved caspase-3 (cat. no. 9661), cleaved caspase-9 (cat. no. 7237), PARP (cat. no. 9542), Cdc2 (cat. no. 28439), Cdc25C (cat. no. 4688), p-IGF1R (Y1131) (cat. no. 9750), IGF1R (cat. no. 3021), PI3K (cat. no. 4292), p-PI3K (cat. no. 4228), AKT (cat. no. 9272), p-AKT (cat. no. 9271), mTOR (cat. no. 2971), nTOR (cat. no. 2972), p-mTOR (cat. no. 2971) (all from Cell Signaling Technology, Beverly, MA, USA) and β-actin (1:5,000; cat. no. YIF-LF-PA0207A; AB Frontier, Seoul, Korea). After washing with Tris-buffered saline and Tween-20 (TBST) thrice, the membranes were incubated for 1 h at room temperature with HRP-labeled secondary antibodies, anti-rabbit IgG (cat. no. 7074) and anti-mouse IgG (cat. no. 7076) diluted to 1:5,000 from Cell Signaling Technology.

Mouse xenograft tumor model. Male BALB/c nude mice at four weeks of age were purchased from Damool Science (Daejeon, Korea). Animals were housed in microisolator ventilated cages with environmentally controlled temperature (21±1°C) and humidity (55±5%), and a reversed 12-h light-dark cycle. Water and food were autoclaved and provided ad libitum. All animal experiments were conducted in accordance with the National Institutes of Health (NIH) Care and Use of Laboratory Animals (21) and were approved by the Chosun University Institutional Animal Care and Use Committee (CIACUC2016-S0040). After one week of acclimatization to the laboratory environment, the mice were subcutaneously inoculated with 0.1 ml of PBS containing A549 cells (1x10⁶ cells/mouse) into the right ear. When the xenograft tumors were palpable and the tumor volume reached ~100 mm³, the mice were randomly assigned to the control and treatment groups (n=3). Mice received either 10% DMSO in normal saline (control) or anthricin (10 and 20 mg/kg body weight) by intraperitoneal injection every three days. The xenograft tumors were excised and weighed.

Statistical analysis. All data were derived from at least three independent experiments (except the in vivo study). Results were expressed as the means ± standard deviation (SD). One-way ANOVA followed by Dunnett’s t-test was employed for multiple comparisons using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA). Statistical significance was set at P<0.05.

Results

Identification of the main components in the methanol extract of dried A. sylvestris roots by HPLC. The chromatograms revealed the presence of anthricin in the methanol extract of A. sylvestris roots (Fig. 1). It was identified by comparing the retention time and UV spectra of the standard sample. The retention time of anthricin was 49.20 min. In Fig. 1B and C, the first peak was ignored as the solvent peak.

Cytotoxic effect of anthricin on A549 cells. To assess the effect of anthricin on cell viability, the cells were treated with anthricin (0, 10, 20, 50, 100 and 200 nM) for the indicated time-points and were analyzed by MTT assay. As shown in Fig. 2, anthricin markedly increased the cytotoxicity of A549 cells in a dose- and time-dependent manner. Since anthricin exhibited cytotoxicity at 10 nM in 24 h, further experiments were performed at 10, 20 and 50 nM.

Induction of apoptosis by anthricin in A549 cells. To determine whether cytotoxicity of anthricin was related to apoptosis, we investigated induction of apoptosis of A549 cells by anthricin using a live and dead assay, DAPI staining, FACS analysis, and western blotting. As shown in Fig. 3A, cells stained with ethidium bromide homodimer-1 (dead dye, red color) gradually increased with anthricin treatment dose-dependently. In addition, anthricin-treated cells exhibited a significant increase in the apoptotic cell population compared with the control. Cells stained brightly by nuclear condensation were considered as apoptotic cells. Based on the apoptosis phenomenon of anthricin observed in the live and dead assay and DAPI staining (Fig. 3A and B, respectively), we performed FACS analysis for the quantification of apoptotic cells. A549 cells were treated with anthricin as previously indicated and then double-stained with PE-Annexin V/7-AAD. The percentage of Annexin V-positive apoptotic cells increased to 13.52, 14.7 and 23.94% at 10, 20 and 50 nM anthricin, respectively, compared with the control (0.09%) (Fig. 3C).

Western blotting was performed to evaluate whether A549 cell apoptosis induced by anthricin was dependent on caspases. As shown in Fig. 3E, the cleavage of caspase-8, -3 and -9 significantly increased in a dose-dependent manner and thus the cleavage of native PARP (116 kDa) into its small fragment PARP (89 kDa) increased. Furthermore, anthricin-mediated apoptosis was associated with the outer mitochondrial membrane in a dose-dependent manner. Anti-apoptotic Bcl-2 protein levels decreased but anti-survival Bax protein levels increased (Fig. 3D). Collectively, these results revealed that anthricin-induced apoptosis of A549 cells may be mediated by the activation of caspase in the extrinsic death receptor and intrinsic mitochondrial-dependent apoptotic signaling pathways.

Induction of cell cycle arrest by anthricin in A549 cells. Flow cytometric analysis revealed that the percentage of cells in the G2/M phase increased in anthricin-treated cells compared with the control (Fig. 4A and B). This peak markedly
increased at 20 and 50 nM anthricin (~81.05 and 68.69%, respectively). In addition, the results for the detected sub-G1 group indicated that anthricin induced apoptosis of A549 cells. Western blot analysis was performed to examine the expression of G2/M-boundary regulatory proteins, including Cdc2 and Cdc25C. As shown in Fig. 4C, the protein expression of Cdc2 and Cdc25C significantly decreased 24 h after anthricin treatment in a dose-dependent manner, indicating that anthricin induces G2/M phase arrest in A549 cells.

Figure 1. HPLC chromatograms of the methanol extract of the dried A. sylvestris root. (A and B) HPLC chromatograms of (A) the methanol extract of the dried A. sylvestris root at 10 mg/ml and (B) prepared anthricin were identified at 210 nm. (C) HPLC chromatograms of the anthricin standard were identified at 210 nm.

Effect of anthricin on the expression of IGF1R, PI3K, and Akt. IGF1R is upregulated in various cancers, including breast, prostate, and lung cancers, and mediates cell cycle progression and prevention of apoptosis in hematopoietic cells (23). Thus, we examined the effects of anthricin on the IGF1R/PI3K/Akt signaling pathways in A549 cells. After 24 h of exposure to anthricin (0, 10, 20 and 50 nM), the protein expression levels of p-IGF1R (Y1131) were reduced to 65, 22 and 3%, at 10, 20 and 50 nM anthricin, respectively (Fig. 5). In addition, anthricin treatment reduced the protein expression levels of p-PI3K (P85) and p-Akt (Ser473), which IGF1R targets downstream in a dose-dependent manner (Fig. 5). As mTOR is a downstream target of the Akt gene, the expression of p-mTOR (Ser2448) was reduced due to the inhibition of Akt (Fig. 5). These results revealed that anthricin induced apoptosis by inhibiting the IGF1R/PI3K/Akt signaling pathways.

Antitumor effect of anthricin in human lung adenocarcinoma cell-derived mouse xenografts in vivo. Based on the aforementioned in vitro anticancer effect, we investigated the inhibitory effects of anthricin on A549 xenografts. Mice were injected (i.p.) with 10 and 20 mg/kg of anthricin every three days for 30 days. The tumors removed from these animals...
Figure 3. Induction of apoptosis by anthricin in A549 cells. Cells were treated with various concentrations of anthricin (0, 10, 20 and 50 nM) for 24 h. (A) Live and dead cells were stained by calcein-AM and ethidium bromide homodimer-1, respectively. Stained cells were observed by fluorescence microscopic analysis and imaged (magnification, x100). (B) After anthricin treatment, DNA was stained with DAPI and observed by fluorescence microscopic analysis and imaged (magnification, x100). (C) Annexin V/7-AAD double-staining revealed the percentage of apoptotic cells after anthricin treatment. The proportion of cells in each quadrant are marked on the figures. (D and E) After anthricin treatment, the expression of apoptotic-related proteins (cleaved caspase-8, cleaved caspase-3, cleaved caspase-9, PARP, Bax and Bcl-2) was assessed by western blotting, and β-actin was used as the loading control. Data are expressed as the means ± SD of three independent experiments.

Figure 4. Effect of anthricin on cell cycle distribution in A549 cells. Cells were treated with various concentrations of anthricin (0, 10, 20, and 50 nM) for 24 h. (A) The cell cycle distribution was monitored by flow cytometry. (B) Data are expressed as the mean ± SD (n=3). (C) The expression of cell cycle-related proteins (Cdc2 and Cdc25C) were assessed by western blotting, and β-actin was used as the loading control. Data are expressed as the means ± SD of three independent experiments. **P<0.001 compared with the control group.
are shown in Fig. 6C, and their size and weight have been provided in Fig. 6C and D, respectively. The tumor growth of A549 xenografts were significantly inhibited by anthricin treatment, and the inhibitory rates were 37.34 and 52.17%, at 10 and 20 mg/kg anthricin, respectively, compared with that in the vehicle-treated animals (Fig. 6B). In addition,
the tumor mean weights were 883.33±41.55, 606.66±18.04 and 496.67±23.84 mg in the control and after treatment with 10 and 20 mg/kg anthricin, respectively; the inhibition rates were 32 and 44% (Fig. 6D). There was no significant difference in body weight between the groups (Fig. 6A) and no sign of skin rash or diarrhea. All animals appeared to be in a decent state despite treatment. These results revealed that anthricin exhibits potential as a novel antitumor therapeutic agent against lung cancer.

**Discussion**

*Anthriscus sylvestris* (L.) Hoffm. has been used in traditional medicine as an antipyretic, analgesic agent and as a cough remedy. It contains lignans such as anthricin (7), which is a major lignan in this plant and exhibits antitumor activity in various cancer cells, such as gastric, breast and lung cancer through apoptosis (13,14). In the present study, we isolated anthricin from *A. sylvestris* and investigated the mechanism underlying apoptosis following anthricin treatment in A549 cells. Previous research has reported that anthricin isolated from *A. sylvestris* inhibits the growth of breast cancer cells by inhibiting Akt/mTOR signaling and enhancing autophagy inhibition (15). In addition, anthricin has demonstrated an anticancer effect through cell cycle arrest at the G2/M phase and caspase-mediated apoptosis in gastric, breast and lung cancer cells (15,24,25).

We found *in vitro* that anthricin treatment induced G2/M phase arrest and caspase-mediated apoptosis in A549 cells. Our data revealed that anthricin treatment strongly inhibited A549 cell viability in a dose- and time-dependent manner and that the suppression of cell viability was due to cell cycle arrest at the G2/M phase. Following 24 h of anthricin exposure, most cells were arrested at the G2/M phase and there was a slight increase in the sub-G1 cell population, as indicated by flow cytometric analysis. Western blot analysis was carried out to examine the mechanism of cell cycle progression and it was revealed that anthricin decreased the expression of Cdc2 and Cdc25C. Cdc2 forms maturation promoting factor (MPF), which regulates the transition from the G2 to the M phase through interaction with cyclin B1 (26). Cdc25C regulates the subsequent activation of the cyclin B1/Cdc2 complex by inhibiting phosphorylation of Cdc2 on Thr14/Tyr15. These results revealed that anthricin induced cell cycle arrest at the G2/M phase by suppressing the cell cycle regulators Cdc2 and Cdc25C. This was consistent with previous studies which revealed that anthricin isolated from *A. sylvestris* inhibits the growth of breast cancer cells by inhibiting Akt/mTOR signaling (15).

In conclusion, this study revealed, for the first time, that anthricin-induced apoptosis was mediated by the IGF1R/Pi3K/Akt signaling pathway in A549 cells. We believe that this study provided meaningful insights. However, the relationship between anthricin and IGF1R warrants further investigation.

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**Availability of data and materials**

The datasets used during the present study are available from the corresponding author upon reasonable request.

**Author’s contributions**

CSK and SMM conceived and designed this study. SMM analyzed the HPLC analysis and prepared sample (anthricin).
BRP and SAL performed the all in vitro experiments. BRP performed the in vivo experiments and wrote the manuscript. CSK reviewed and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of this research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All experimental protocols were approved by the Institutional Animal Care and Use Committee of Chosun University (CIACUC2016-S0040).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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