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Antcin K from the medicinal mushroom Antrodia cinnamomea attenuates cell migration and epithelial-mesenchymal transition in lung adenocarcinoma

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Antcin K from the medicinal mushroom *Antrodia cinnamomea* attenuates cell migration and epithelial-mesenchymal transition in lung adenocarcinoma

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Abstract

Lung cancer stands as a leading cause of cancer-related deaths worldwide, with a high mortality rate due to late-stage metastasis. The epithelial-mesenchymal transition (EMT) process plays a crucial role in cancer progression, contributing to the invasive and metastatic traits observed in lung cancer. *Antrodia cinnamomea*, a Taiwanese mushroom, has shown diverse biological activities including hepatoprotection and anti-inflammatory responses, as well as anticancer properties. However, research on its efficacy against lung cancer metastasis is limited. In current study, we found the potential of Antcin K, a predominant compound in *Antrodia cinnamomea*, to inhibit the migratory potential of lung cancer cells. Firstly, the cell viability assay was used to explore the cytotoxicity effect of Antcin K in A549 and CL1-0 lung cancer cells. The results showed that Antcin K had no cytotoxicity effect under 40 μM. We investigated the anti-EMT potential of Antcin K in lung cancer cells, and results showed that pretreatment with Antcin K dramatically inhibited transforming growth factor-beta (TGF-β)-induced mesenchymal-like change of lung cancer cells. Furthermore, pre-treatment with Antcin K also abolished cell migration ability in response to TGF-β stimulation. Finally, we found Antcin K could reduce vimentin expression in response to TGF-β stimulation in lung cancer cells. In summary, our current study highlights the potential of Antcin K to prevent metastasis of lung cancer by targeting EMT, offering a novel therapeutic approach for lung cancer treatment.

Key words: Lung adenocarcinoma, epithelial-mesenchymal transition, cancer metastasis, *Antrodia cinnamomea*, antcin k

Introduction

Lung cancer ranks as the primary cause of global cancer-related mortality. Approximately 85% of lung cancer cases are classified as non-small cell lung cancer (NSCLC) [1]. Currently, the 5-year survival rate post-diagnosis is around 15%, mostly attributed to metastasis in late-stage of lung cancer, which significantly contributes to the poor prognosis of the disease. The precise mechanisms governing metastasis in this context remain poorly understood.

Abundant evidence underscores the pivotal role of the epithelial-mesenchymal transition (EMT) process in comprehending the invasive and metastatic characteristics inherent in cancer progression [2]. EMT is characterized by the loss of apical-basal polarity in epithelial cells, leading to their transition into a mesenchymal phenotype [3]. Notably, extensive investigations have revealed EMT-like states in lung cancer, with the activation of specific transcription factors such as Slug being implicated in conferring invasive behaviors to lung cancer cells [4].

Antrodia cinnamomea, a mushroom found in Taiwan, has attracted attention for its multifaceted biological activities. Research indicates its efficacy in hepatoprotection, anti-inflammatory responses [5], and anti-hepatitis C virus activity [6]. Additionally, *Antrodia cinnamomea* has demonstrated promising anticancer properties, making it a compelling subject for further exploration in the field of medicine [7]. Antcin K, the predominant ergostane triterpenoid found in the fruiting bodies of *Antrodia cinnamomea* cultivated on basswood, has been identified as a potent anti-tumoral agent by inducing cancer cell apoptosis [8], and inhibiting cancer cell mobility [9], there is a notable absence of research on its effectiveness in preventing metastasis in lung cancer cells. In current study, we show the promising effect of Antcin K on cell migratory potential in lung cancer cells. This phenomenon may be exerted by targeting EMT. This work provides a novel application of Antcin K in lung cancer therapy.

Materials and Methods

Material

Anti-mouse and anti-rabbit IgG-conjugated horseradish peroxidase, rabbit polyclonal antibodies specific for vimentin and β-actin were purchased from Cell Signaling Technology (Danvers, MA, USA.) and Sigma–Aldrich (St Louis, MO, USA). All other chemicals were obtained from Sigma–Aldrich (St Louis, MO, USA).

Cell culture

The A549 human lung adenocarcinoma cell line was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Additionally, the CL1-0 human lung adenocarcinoma cell lines, were generously provided by Dr. Shun-Fa Yang from the Institute of Medicine, Chung Shan Medical University, Taichung, Taiwan. A549 cells were grown in F12-K medium, while CL1-0 cells were maintained in α-MEM medium. To make the complete media, the indicated media were supplied with 20 mM HEPES, 10% FBS, 2 mM glutamine, penicillin, and streptomycin (Invitrogen, Carlsbad, CA). The cells were maintained at 37°C in a 5% CO_2 atmosphere.

Cell viability assay

To determine cell viability of lung cancer cells in response to Antcin K treatment, the resazurin cell viability assay was conducted (Biotium; Fremont, CA, USA). The lung cancer cells with 2 × 10^4 density was seeded in 96-well plates and exposed them to varying concentrations of Antcin K. After 24 hours, the media were removed and added 100 μL of fresh media with resazurin solution (10 μL). The plates were then kept in the dark at 37°C for an additional 1 hours. The cell viability was assessed by differences in fluorescence emission (Em: 590 nm) using an excitation wavelength of 540 nm.

Cell morphology

To observe EMT alterations in cell culture, we conducted a cell scatter assay on lung cancer cells. The cells were initially seeded in a 24-well plate at a density of 0.5 × 10^4 cells per well, forming compact colonies within 48 hours. Subsequently, the cells underwent specific treatments as detailed in the figure legends, followed by an additional 24 hours incubation period. The cells were then fixed using 4% paraformaldehyde (PFA) and captured under a microscope-mounted camera.

Transwell migration assay

All cell migration assays were performed using Transwell inserts (8-μm pore size; Costar, NY) in 24-well plates. The lung cancer cells (1 × 10^4 in 200 μl of serum-free medium) were then seeded in the upper chamber of the Transwell in the presence of the indicated concentrations of Antcin K or vehicle (0.1% DMSO). Three hundred μl of the

same medium was placed inside 24-well. Twenty-four hours after incubation, the cells on Transwell were using 4% PFA and stained with 0.05% crystal violet. The cells inside Transwell which didn't migrate across the membrane were removed by Q-tips. Finally, the cells migrated across the membrane were photographed using microscope. Each experiment was performed more than triplicate.

Wound healing assay

In wound-healing migration assay, the Culture-Inserts purchased from Ibidi (Gräfelfing, Germany) were used to measure cell migration. The cells were seeded to each well at a concentration of 1 × 10^5 cells in the culture medium. After 24 hours, a complete cell-free gap was observed by removal of the Culture-Insert. Images were captured at 0 h and 24 h using microscope. The percent of wound closure was calculated with NIH ImageJ software.

Western Blot analysis

To study protein expression level, cell extracts were prepared, separated proteins using SDS-PAGE, and transferred them onto Immobilon polyvinyldifluoride (PVDF) membranes. The membranes were treated with a blocking agent (4% BSA) for an hour at room temperature to prevent non-specific interactions. After that, we used rabbit or mouse anti-human antibodies to identify vimentin and β-actin (1:1000) for another hour at room temperature. After three washes, the blots were incubated with a donkey anti-rabbit peroxidase-conjugated secondary antibody (1:1000) for an hour at room temperature. Finally, the signals on the blots were then visualized using enhanced chemiluminescence and captured with ImageQuant LAS 4000 (GE Healthcare Life Sciences, Little Chalfont, UK). Quantitative data analysis was performed using NIH ImageJ software.

Quantitative polymerase chain reaction

Cells cultured in 6-well plates were utilized for quantitative polymerase chain reaction (qPCR) analysis. Total RNA was extracted from the treated cells using easy-BLUE™ Total RNA Extraction Kit (iNtRON Biotechnology Inc., Seongnam, Kyonggi-do, South Korea) according to the manufacturer's protocol, and RNA quality was assessed by RNA gel electrophoresis. The RNA concentration was measured using the NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA),

followed by reverse transcription of 1 μg mRNA to produce complementary DNA (cDNA).

For qPCR, the KAPA PROBE FAST qPCR Kit Master Mix (Sigma-Aldrich) was employed, with qPCR probes obtained from Applied Biosystems™ (Waltham, MA, USA). The qPCR reactions were performed in triplicate on a StepOnePlus instrument (Applied Biosystems), with cycling conditions set at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the reference gene for normalization. The relative RNA expression levels of the target gene were determined using the 2−(ΔΔCT) method.

Statistical analysis

All statistical analyses were conducted using GraphPad Prism 8 (GraphPad, USA). The values are reported as means ± standard errors of the means. Statistical comparisons were performed using one-way analysis of variance (ANOVA), followed by Fisher's least significant difference (LSD) post hoc test. In all the comparisons, $p < 0.05$ was considered significant. * represents $p < 0.05$, ** represents $p < 0.01$, *** represents $p < 0.001$, **** represents $p < 0.0001$.

Results

Antcin K did not have a significant cell cytotoxicity effect

At first, we assessed the nontoxic dose of Antcin K in lung cancer cells by resazurin cell viability assay. The A549 and CL1-0 lung cancer cells were incubated with different dose of Antcin K (0-40 μM). The data indicated that Antcin K did not have significant cell cytotoxicity effect in both lung cancer cells under 40 μM concentration (Fig. 1).

Antcin K reduces mesenchymal-like morphologic change in response to TGF-β induction in lung cancer cells

Previous study revealed that Antcin K affected EMT-related proteins expression [9], suggesting its anti-metastatic potential in liver cancer. The morphological change of lung cancer cells was monitored to confirmed the EMT change after Antcin K treatment. In our results, TGF-β exposure dramatically induced mesenchymal-like morphologic change of both lung cancer cells, while co-treatment with Antcin K obviously reversed this phenomenon (Fig. 2). The results reveal that Antcin K is able to inhibit EMT change

in lung cancer cells.

Antcin K abrogates cell migratory potential in response to TGF-β induction in lung cancer cells

The EMT change is associated with enhanced cell migration ability [10]. The Transwell migration assay was performed to examine the anti-migratory effect of Antcin K in lung cancer cells. The results showed that treatment of Antcin K significantly inhibited cell migration in both A549 and CL1-0 lung cancer cells whereas alone or in TGF-β induction group (Fig. 3). Furthermore, the wound healing assay was conducted to confirmed cell migration potential as well. In accordance with the result of Transwell migration, treatment of Antcin K obviously reduced cell migrated across the wound which was caused by TGF-β induction (Fig. 4). These results showed that Antcin K inhibited EMT-related cell migration in lung cancer cells.

Antcin K inhibits vimentin expression in response to TGF-β induction in lung cancer cells

Finally, we investigated vimentin expression, which is known as a key EMT marker for mediating metastasis [11]. The protein expression of vimentin was increased following TGF-β stimulation; however, this effect was abolished after Antcin K treatment (Fig. 5A). The data of mRNA expression of vimentin was similar to protein expression (Fig. 5B). According to these findings, TGF-β-induced EMT in lung cancer cells can be reversed by Antcin K treatment.

Discussion

Antcin K, a triterpenoid isolated from the medicinal mushroom *Antrodia cinnamomea* found in Taiwan, has been extensively studied for its diverse biological activities [12]. Previous studies have demonstrated its notable inhibitory effects on cancer cell growth and its ability to induce apoptosis in various cancer cell lines [8, 13]. Understanding the molecular mechanisms underlying its anti-tumor functions could develop novel therapeutic strategies against cancer. In our investigation, we observed that Antcin K effectively impeded the migratory capabilities of lung cancer cells, consistent with earlier findings [9]. In this study, Antcin K effectively inhibited the adhesion, migration, and invasion of Hep 3B cells [9]. Intriguingly, *Antrodia cinnamomea* extract has been shown to reduce migration of lung cancer cells by targeting MMP2 and MMP9 [14].

Additionally, *Antrodia cinnamomea* extract has demonstrated anti-migratory effects in human adenocarcinoma CL1-0 cells via the MAPK and PI3K/AKT signaling pathways [15]. Our results align with these previous findings, revealing the potential of *Antrodia cinnamomea* and its bioactive compound, Antcin K, as therapeutic agents for cancer metastasis. A study by Wu et al. [16] demonstrated that the ethanolic extract of *Antrodia cinnamomea* suppressed cell growth, migration, and drug resistance in human lung cancer A549 cells and a C57BL/6J allograft tumor model. The anti-metastatic effect of Antcin K should be investigated in the future.

EMT plays a pivotal role in tumor metastasis by inducing a phenotypic switch in cancer cells. During EMT, epithelial cells lose their cell-cell adhesion properties and acquire mesenchymal characteristics, allowing them to invade surrounding tissues, intravasate into blood or lymphatic vessels, and colonize distant organs [17]. During EMT, epithelial cells downregulate epithelial markers such as E-cadherin (E-cad) and upregulate mesenchymal markers like vimentin, facilitating the loss of cell-cell adhesion and gaining of mesenchymal traits [18]. Here, we found Antcin K could reverse TGF-β-induced EMT in lung cancer cells, which may be regulated by vimentin down-regulation. Previous have indicated that *Antrodia cinnamomea* extract can inhibit EMT in various cancer cell lines. Specifically, the *Antrodia cinnamomea* extract has been shown to suppress Wnt/β-catenin, a crucial EMT pathway involved in EMT process, which in turn reversing the EMT process and diminishing the invasive and migratory capabilities of colon cancer cells [19]. In triple-negative breast cancers, *Antrodia cinnamomea* extract reversed EMT by upregulating E-cadherin expression and inhibiting Twist expression. AC treatment also decreased Wnt/β-catenin nuclear translocation through a GSK3β-dependent pathway and downregulated mesenchymal markers while upregulating epithelial markers [20]. Previous study indicated that Antcin K downregulated vimentin, and upregulated E-cadherin, thus contributing to inhibition of liver cancer metastasis [9]. Conclusively, these findings suggest that Antcin K is a major component of *Antrodia cinnamomea* extract responsible for its anti-metastatic effect by targeting EMT.

Conclusions

In the present study, we discovered that Antcin K can diminish the migratory capacity of lung cancer cells by targeting the EMT marker, vimentin, highlighting a novel clinical application for Antcin K in lung cancer therapy.

Declaration

Acknowledgement

Not applicable

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Availability of data and materials

The data sets used and analyzed during the current study are available from the corresponding author on reasonable request.

Author contribution

Study conception and design: C.C. Chao, S. Y. Hsiao, P.C. Chen; data collection: C.C. Chao; analysis and interpretation of results: C.C. Chao; draft manuscript preparation: P.C. Chen, M.L. Hsu; funding acquisition: M. H. Yen, S. Y. Hsiao, W. C. Kao; Resources: Y. H. Kuo, C. H. Tang, C. Y. Lin

Ethical Approval and consent to participate

Not applicable

Patient consent for publication

Not applicable

Competing interests

The authors state no conflict of interest

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Figure 1

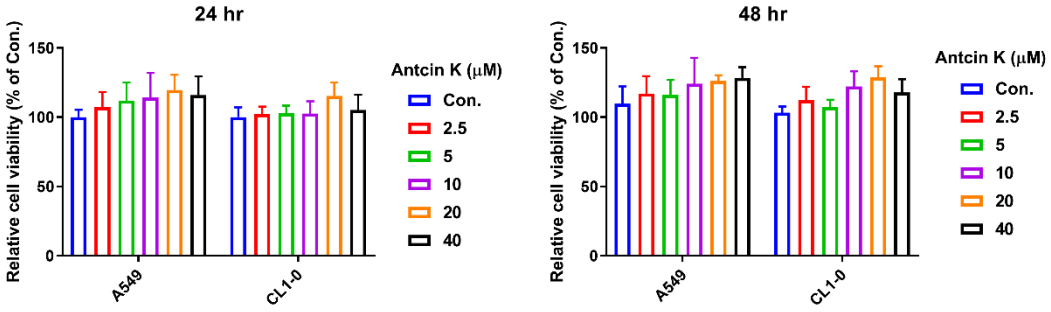

24 hr
Relative cell viability (% of Con.)
150
100
50
0
A549
CL1-0
Antcin K (μM)
Con.
2.5
5
10
20
40
48 hr
Relative cell viability (% of Con.)
150
100
50
0
A549
CL1-0
Antcin K (μM)
Con.
2.5
5
10
20
40

Figure 2

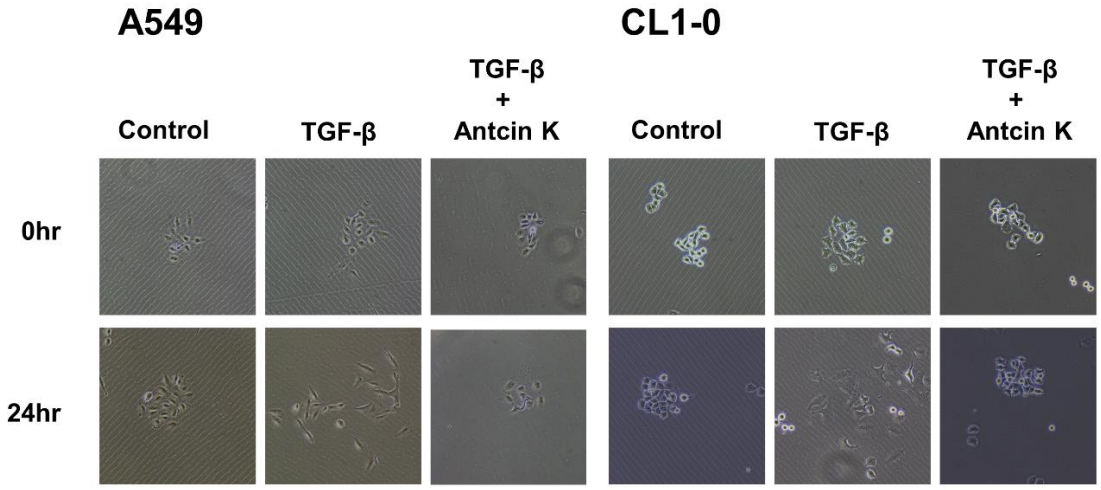

A549
CL1-0
Control
TGF-β
TGF-β + Antcin K
Control
TGF-β
TGF-β + Antcin K
0hr
24hr

Figure 3

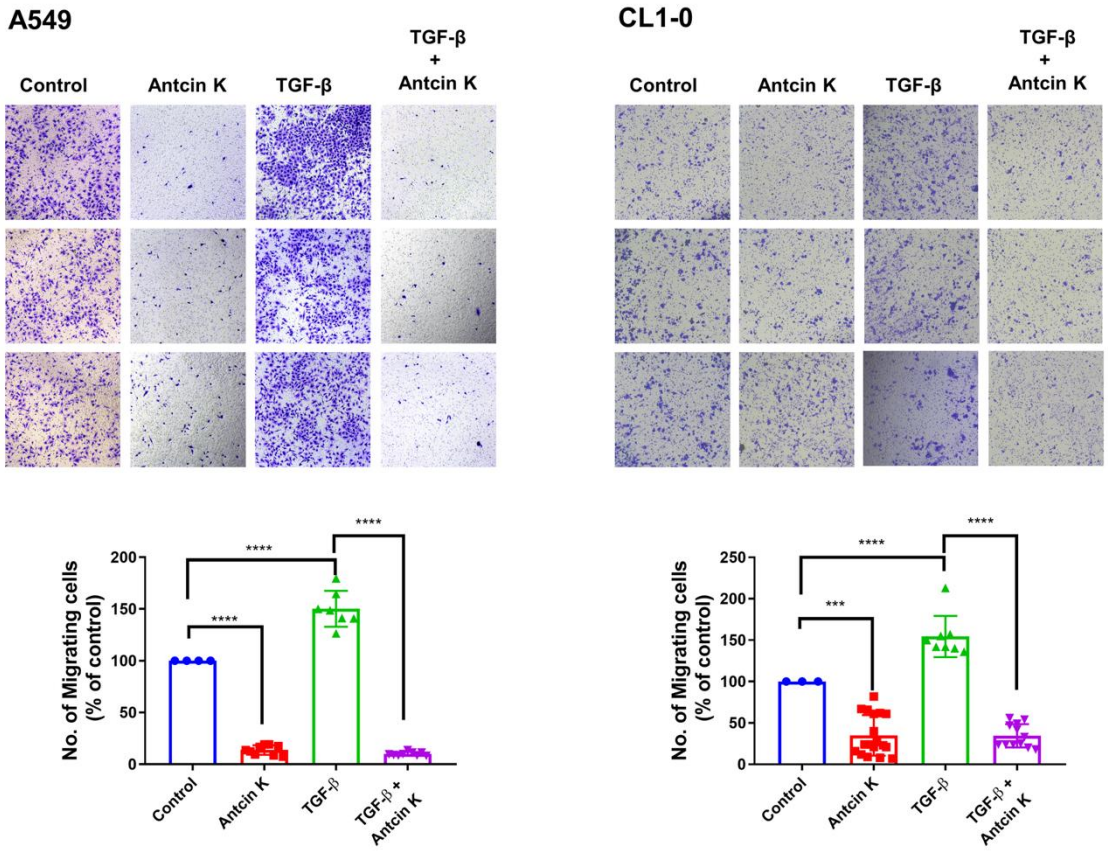

A549
Control
Antcin K
TGF-β
TGF-β + Antcin K
No. of Migrating cells (% of control)
****
****
****
Control
Antcin K
TGF-β
TGF-β + Antcin K
CL1-0
Control
Antcin K
TGF-β
TGF-β + Antcin K
No. of Migrating cells (% of control)
***
****
****
Control
Antcin K
TGF-β
TGF-β + Antcin K

Figure 4

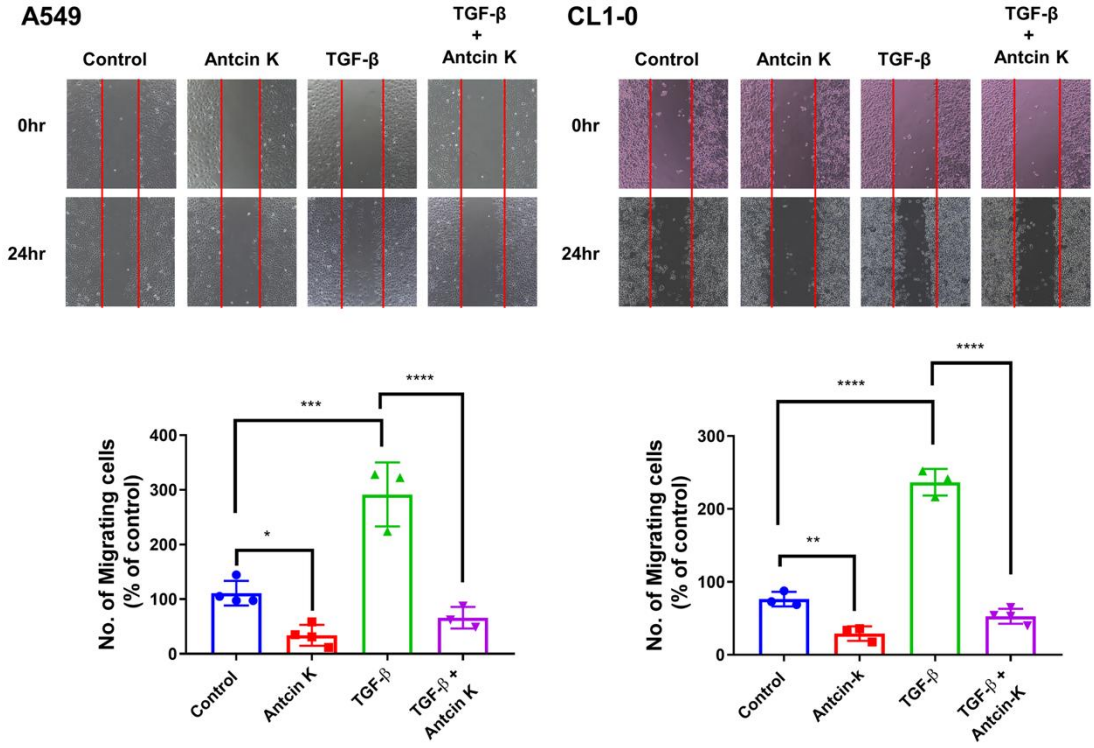

A549
Control
Antcin K
TGF-β
TGF-β + Antcin K
0hr
24hr
CL1-0
Control
Antcin K
TGF-β
TGF-β + Antcin K
0hr
24hr
No. of Migrating cells (% of control)
400
300
200
100
0
*
***
****
Control
Antcin K
TGF-β
TGF-β + Antcin K
No. of Migrating cells (% of control)
300
200
100
0
**
****
****
Control
Antcin-k
TGF-β
TGF-β + Antcin-K

Figure 5

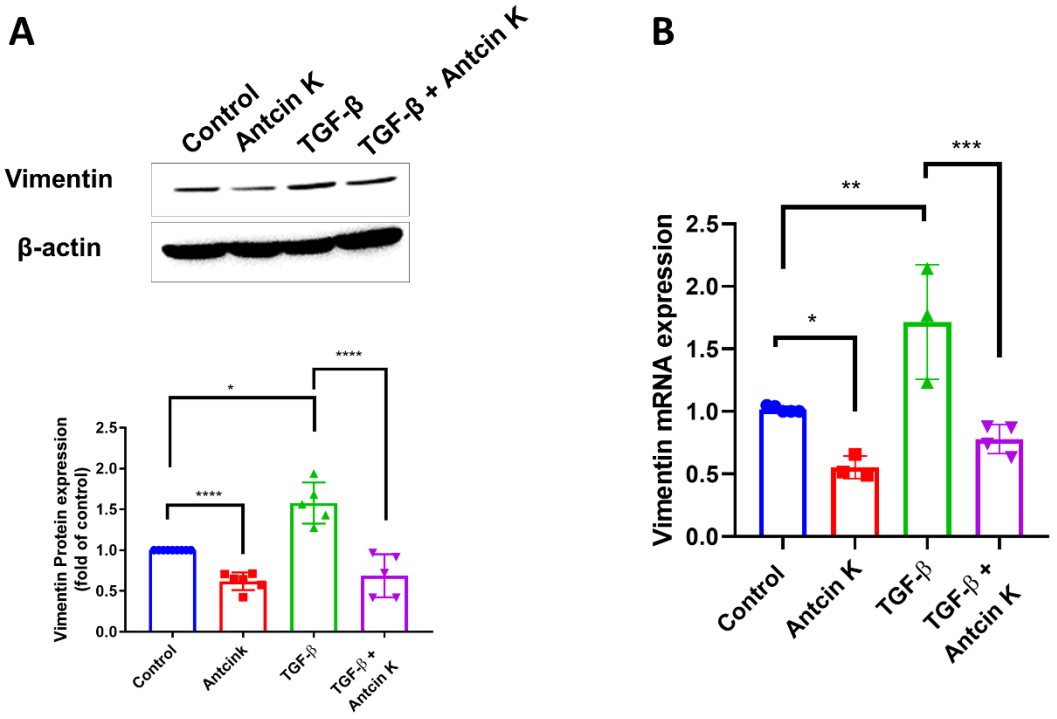

A
Control
Antcin K
TGF-β
TGF-β + Antcin K
Vimentin
β-actin
Vimentin Protein expression (fold of control)
2.5
2.0
1.5
1.0
0.5
0.0
*
****
****
Control
Antcink
TGF-β
TGF-β + Antcin K
B
Vimentin mRNA expression
2.5
2.0
1.5
1.0
0.5
0.0
*
**
***
Control
Antcin K
TGF-β
TGF-β + Antcin K

Figure legends

Figure 1. The cell viability assay of lung cancer cells in response to Antcin K treatment.

The A549 and CL1-0 lung cancer cells were incubated with different concentrations of Antcin K (0, 2.5, 5, 10, 20, 40 μM) for 24 h or 48 h. The cell viability was determined by resazurin assay.

Figure 2. Antcin K inhibits TGF-β-induced EMT in lung cancer cells.

The A549 and CL1-0 lung cancer cells were stimulated with TGF-β (20 ng/mL) in the presence of Antcin K (20 μM) or vehicle control for 24 h. The cellular morphology was observed through a microscope.

Figure 3. Antcin K prevents TGF-β-induced migration ability in in lung cancer cells.

The Transwell migration assay was used to assess the cell migration ability. A549 and CL1-0 lung cancer cells were stimulated with TGF-β (20 ng/mL) in the presence of Antcin K (20 μM) or vehicle control for 24 h in Transwell. The cells that had migrated across the membrane were stained and photographed using a microscope, and the number of migrated cells was quantified in the lower panel. The results are expressed as mean ± SEM, with ***$p < 0.001$, and ****$p < 0.0001$ indicating significant differences compared to each group.

Figure 4. Antcin K blocks TGF-β-induced wound healing in in lung cancer cells.

The wound healing assay was used to assess the cell migration ability. A549 and CL1-0 lung cancer cells were stimulated with TGF-β (20 ng/mL) in the presence of Antcin K (20 μM) or vehicle control for 24 h in 24 well. The cells that had migrated across the wound photographed using a microscope at 0 h and 24 h, and the number of migrated cells was quantified in the lower panel. The results are expressed as mean ± SEM, with *$p < 0.05$ and **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ indicating significant differences compared to each group.

Figure 5. Antcin K abolishs TGF-β-induced EMT in in lung cancer cells.

A549 lung cancer cells were stimulated with TGF-β (20 ng/mL) in the presence of Antcin K (20 μM) or vehicle control for 24 h. (A) Total cell lysate was collected, and a Western blot was conducted to detect Vimentin expression. β-actin was used as internal control. The quantification results of the Western blot are presented in the lower panel. (B) Total RNA was extract, and a qPCR was conducted to detect Vimentin expression. The results are expressed as mean ± SEM, with *$p < 0.05$ and **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ indicating significant differences compared to each group.