Am J Cancer Res 2025;15(1):59-68
www.ajcr.us /ISSN:2156-6976/ajcr0161894

Original Article

Hinokitiol reduces tumor metastasis by regulating epithelial cell adhesion molecule via protein kinase-B/mammalian target of rapamycin signaling pathway

Pei-Shan Wu[1*], Jing-Ru Weng[2*], Shih-Han Chiu[3], Li-Hsien Wu[2,3], Pei-Hsuan Chen[3], Yun-Xuan Wang[3], Po-Yen Chiu[3], Che-Hsin Lee[3,4,5,6,7]

[1]Department of Otolaryngology, Kaohsiung Armed Forces General Hospital, Kaohsiung, Taiwan; [2]Department of Marine Biotechnology and Resources, National Sun Yat-sen University, Kaohsiung, Taiwan; [3]Department of Biological Sciences, National Sun Yat-sen University, Kaohsiung, Taiwan; [4]Aerosol Science Research Center, National Sun Yat-sen University, Kaohsiung, Taiwan; [5]College of Semiconductor and Advanced Technology Research, National Sun Yat-sen University, Kaohsiung, Taiwan; [6]Department of Medical Laboratory Science and Biotechnology, Kaohsiung Medical University, Kaohsiung, Taiwan; [7]Department of Medical Research, China Medical University Hospital, China Medical University, Taichung, Taiwan. [*]Equal contributors.

Received November 10, 2024; Accepted December 28, 2024; Epub January 15, 2025; Published January 30, 2025

Abstract: Tumor metastasis is the leading cause of death in cancer patients. Epithelial cell adhesion molecule (EpCAM) is abundantly expressed in various malignant tumors and plays a crucial role in cell adhesion, metastasis, proliferation, and differentiation. This study investigated the effects of hinokitiol, a natural tropolone compound known for its antiviral, anti-inflammatory, and antibacterial properties, on tumor growth and metastasis. Specifically, the study focused on the expression of EpCAM in mouse tumor cells treated with hinokitiol. Hinokitiol was administered to mouse melanoma cells (B16F10) and mouse colorectal carcinoma cells (CT26), resulting in a significant decrease in EpCAM expression. Additionally, the protein levels involved in the protein kinase-B/mammalian target of rapamycin (AKT/mTOR) signaling pathway were reduced following hinokitiol treatment. Using wound healing and Transwell assays, the study demonstrated that hinokitiol effectively inhibits cancer cell migration. *In vivo* experiments were conducted using mice, which were injected intravenously with B16F10 or CT26 cells to induce tumor metastasis. The tumor cells were either treated with hinokitiol or left untreated. The results showed that tumor cells treated with hinokitiol exhibited significantly reduced tumor size and weight in the lungs, as well as prolonged survival, compared to untreated tumor cells. This study concludes that hinokitiol inhibits tumor migration by downregulating EpCAM via the AKT/mTOR signaling pathway and exhibits positive effects *in vivo*.

Keywords: Hinokitiol, epithelial cell adhesion molecule, tumor migration, metastasis

Introduction

Cancer incidence is steadily increasing globally, positioning it as the leading cause of death worldwide. Among cancer-related fatalities, metastasis is the primary driver. This complex process involves tumor cells spreading via the bloodstream or lymphatic system, forming circulating tumor cells (CTCs). Metastasis not only complicates treatment but also contributes to multiple organ failure and mortality in advanced stages, making it a critical factor in cancer-related deaths [1].

Tumor metastasis is regulated by a network of signaling pathways and factors, including transforming growth factor-beta (TGF-β), epidermal growth factor (EGF), and hepatocyte growth factor (HGF). These extracellular signals activate transcription factors such as Snail, ZEB1, ZEB2, TWIST1, and Smad2/3, which promote epithelial-mesenchymal transition (EMT) and enhance tumor progression [2]. A key player in metastasis is the epithelial cell adhesion molecule (EpCAM), a type I transmembrane glycoprotein highly expressed on epithelial tissues and various malignant tumors. Initially identified as an

https://doi.org/10.62347/UZFZ9554

adhesion molecule, EpCAM is now recognized for its roles in tumor proliferation, metastasis, invasion, differentiation, and poor prognosis [3]. It is considered a tumor-associated antigen and a marker for CTC detection [4].

Research has revealed that EpCAM undergoes proteolytic cleavage by metalloproteinases like presenilin 2 (PS-2), releasing its extracellular domain (EpEX) and intracellular domain (EpICD), which regulate downstream signaling pathways [5]. These pathways include Akt/mTOR, Ras/Raf/ERK, and Wnt/β-catenin, all of which influence tumor progression [6-8]. In epithelial cells, EpCAM disrupts the interaction between α-catenin and F-actin, inhibiting E-cadherin-mediated adhesion [9]. Additionally, EpEX interacts with EGFR and HGFR, inducing EMT-related genes, enhancing cancer cell migration, and stabilizing PD-L1 protein to promote immune evasion [10]. Given EpCAM's role in metastasis, targeting its activity through protein interactions or signaling pathways is a promising therapeutic strategy. This study focuses on hinokitiol's potential to inhibit EpCAM expression and suppress metastasis in mouse melanoma and colorectal cancer cells.

In recent years, natural medicines have emerged as effective treatments for various diseases, including cancer, by targeting multiple signaling pathways [11, 12]. Due to the limitations of conventional therapies, such as side effects and drug resistance, natural compounds are viewed as safer alternatives. Combining natural medicines with chemotherapy has shown promise in enhancing efficacy, reducing side effects and drug resistance, and preventing recurrence and metastasis [13]. Hinokitiol, a natural monoterpenoid extracted from the heartwood of Taiwan's Cupressaceae plants, is widely used in health products like cosmetics, toothpaste, and food as an antimicrobial agent. Beyond these applications, hinokitiol exhibits pharmacological properties, including anti-inflammatory, antimicrobial, anticancer, antioxidant, and neuroprotective effects [14-16]. Recent studies demonstrate that hinokitiol can induce ferroptosis, promote reactive oxygen species (ROS)-mediated apoptosis, and suppress cell growth in triple-negative breast cancer [17, 18]. Moreover, hinokitiol has been shown to reduce metastasis and modulate drug resistance via the Akt/mTOR pathway [11]. These findings underscore its potential as an anti-metastasis agent, warranting further investigation into its use as a targeted therapy for tumors.

Materials and methods

Reagents, cell lines, plasmids, and mice

Hinokitiol and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The constitutively active AKT plasmid was generously provided by Dr. Chiau-Yuang Tsai (Department of Molecular Immunology, Osaka University, Japan). B16F10 (mouse melanoma) [19], CT26 (mouse colon cancer) [3], 4T1 and LL2 cell lines were cultured in a medium containing 2 mM l-glutamine, 1% antibiotics, and 10% heat-inactivated fetal bovine serum (FBS) at 37°C in a 5% CO_2 atmosphere. Female C57BL/6 (for the B16F10 mouse tumor model) and BALB/c (for the CT26 mouse tumor model) mice were purchased from the Taiwan Laboratory Animal Center. All experimental protocols adhered to the Animal Protection Act of Taiwan and were approved by the Laboratory Animal Care and Use Committee of National Sun Yat-sen University.

Cell viability

Tumor cells were treated with varying concentrations of Hinokitiol (0, 0.125, 1.25, 12.5 μM) for 16 hours, and cell viability was assessed using the WST-1 assay [20].

Immunoblotting and transfection

B16F10 and CT26 cells were seeded in 6-well plates at a density of 4×10^5 cells/well. After 24 hours, the cells were treated with hinokitiol for 16 hours, followed by the addition of 100 μL of lysis buffer to each well. Cells were lysed for 30 minutes and then centrifuged at 12,000 rpm for 10 minutes. The supernatant was collected, and protein content was determined using the bicinchoninic acid (BCA) protein assay (Pierce Biotechnology, Rockford, IL, USA). Cytosolic protein samples (40 μg) were subjected to 8% or 10% SDS-PAGE and then transferred to Hybond-enhanced chemiluminescence nitrocellulose membranes (Amersham, Little Chalfont, UK). The membranes were blocked and incubated with primary antibodies at 4°C overnight, followed by incubation with second-

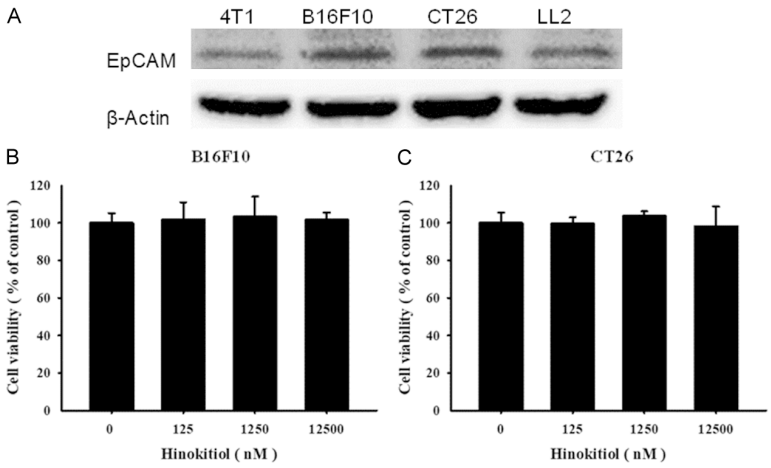

Figure 1. The epithelial adhesion molecule (EpCAM) expression in various murine tumor cells and effect of hinokitiol on B16F10 and CT26 cells. The expression levels of EpCAM in various cancer cell lines. (A) The levels of EpCAM proteins in various cancer cell lines. B16F10 and CT26 cells were cultured in a 96-wells plate with a number of 10^4, and then B16F10 and CT26 cells were treated with different concentrations of hinokitiol (0, 0.125, 1.25, 12.5 μM) for 16 hours. The viability of (B) B16F10 and (C) CT26 cells was determined using the WST-1 method. *, P<0.05; **, P<0.01; ***, P<0.001 (Mean ± SD, n = 4).

ary antibodies. Protein-antibody complexes were detected using an enhanced chemiluminescence system (Amersham), and signals were quantified using ImageJ software [21].

Wound healing assays

Wound healing assays were conducted according to the manufacturer's instructions (IBIDI, Martinsried, Germany). Cells were seeded in Culture-Insert 2 wells at a density of 10^4 cells/well. After cell attachment, a gap was created to observe cell movement. Migration distances were measured under a microscope at 0, 12, and 24 hours. The migration distance of control cells (0 μM) was set as 100% for reference, and these values were compared with those of cells treated with Hinokitiol (12.5 μM) [22].

Transwell assays

For Transwell assays, 24-well inserts (8.0 μm Millicell® Hanging Cell Culture Inserts) were placed in FBS-DMEM wells containing 500 μL of FBS. Cells (5×10^3-10^4, 200 μL/well) were seeded into the inserts and incubated at 37°C in 5% CO_2 for 24 hours. The inserts were then washed with PBS and placed in wells containing DMEM, followed by treatment with either control (0 μM) or Hinokitiol (12.5 μM) for 16 hours. Inserts were washed with PBS, fixed in formaldehyde for 3 minutes, stained with 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) for 1 minute, and observed under a fluorescence microscope for cell counting [23].

Animal studies

C57BL/6 mice were inoculated with 1.25 μM hinokitiol-treated or untreated B16F10 via the tail vein. Meanwhile, BALB/c mice were also treated with 12.5 μM hinokitiol-treated or untreated CT26. Mice mortality was recorded after tumor inoculation. C57BL/6 or BALB/c mice were inoculated with hinokitiol-treated or untreated tumor cells as already described. After 15 days, mice were sacrificed and dissected to observe lung tumor and record tumor weight. Lung tissue samples were fixed in 3.7% formaldehyde and embedded in paraffin for sectioning and histochemical staining. Tissue sections were then microscopically examined for the presence of tumor nodules [23].

Statistical analysis

Differences between groups were analyzed using Student's t-test. Survival analyses were performed using Kaplan-Meier survival curves and the log-rank test. *P*-values less than 0.05 were considered statistically significant.

Results

Effect of hinokitiol on B16F10 and CT26 cells

EpCAM is overexpressed in tumor cells. Initially, EpCAM protein levels were assessed in various murine cancer cell lines. B16F10 and CT26 cells exhibited greater EpCAM expression levels than the other murine tumor cells (**Figure 1A**). The B16F10 cell line, derived from murine melanoma, is widely recognized for its aggressive metastatic phenotype. These cells exhibit a strong ability to invade surrounding tissues, intravasate into blood vessels, and form sec-

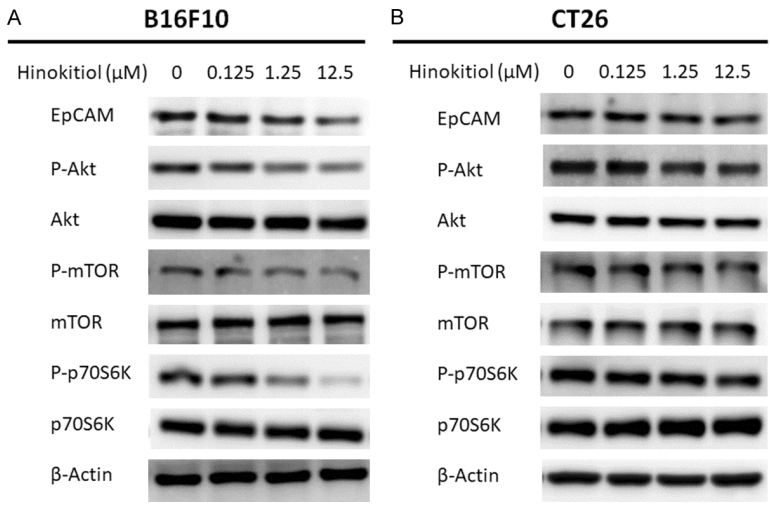

Figure 2. Hinokitiol treatment of B16F10 and CT26 cells, expression of EpCAM and AKT/mTOR/p70S6K pathway. B16F10 and CT26 cells were cultured in 6-well plates at 3×10^5 and 4×10^5, respectively. Hinokitiol (0, 0.125, 1.25, 12.5 μM) were added for treatment for 16 hours. The cells were collected and extracted. Western blotting was used to determine whether the expression levels of EpCAM and AKT/mTOR/p70S6K-related proteins after Hinokitiol treatment were dose-dependent. The β-actin expression served as loading controls for and total protein. Each experiment was repeated three times with similar results. *, P<0.05; **, P<0.01; ***, P<0.001 (Mean ± SD, n = 3).

ondary tumors, particularly in the lungs. The high metastatic potential of B16F10 makes it an ideal model for studying melanoma progression, metastasis mechanisms, and evaluating anti-metastatic therapies. The CT26 cell line, derived from murine colorectal carcinoma, also displays metastatic properties, though typically less aggressive than B16F10 cells. CT26 cells frequently metastasize to the liver and lungs when introduced into experimental models. This cell line is commonly used to study colorectal cancer metastasis, immune responses, and the efficacy of therapeutic agents targeting metastatic colorectal cancer. Both cell lines are valuable in cancer research for their ability to mimic human cancer metastasis processes and serve as platforms to evaluate the effects of therapeutic interventions aimed at inhibiting metastasis. Since hinokitiol can influence protein levels in tumor cells, we treated tumor cell lines to hinokitiol at different concentrations [23]. Hinokitiol did not significantly affect the cell viability at the highest dose for 16 hours. This ensures that cell survival is not compromised by hinokitiol's potential toxicity. As shown in **Figure 1B** and **1C**, different concentrations of hinokitiol (0, 0.125, 1.25, 12.5 μM) were treated with B16F10 and CT26 cells for 16 hours. It was confirmed that these two cell lines were not significantly affected in terms of survival rate at concentrations up to 12.5 μM.

Effects of hinokitiol on EpCAM and the Akt/mTOR/p70S6K pathway in B16F10 and CT26 cells

Previous studies have indicated that hinokitiol can regulate downstream proteins via the Akt/mTOR signaling pathway [24]. Additionally, some studies suggest that EpCAM can be regulated through this pathway. To explore this, Western blot analysis was conducted to examine the Akt/mTOR signaling and EpCAM protein expression in B16F10 and CT26 cells. The results in **Figure 2** demonstrate that as the concentration of hinokitiol increased (0-12.5 μM) in two cells, the expression levels of EpCAM, phospho-Akt, phospho-mTOR and phodpho-p70S6K proteins decreased in a dose-dependent manner (Figures S1 and S2).

Constitutively active-Akt reduces the effect of hinokitiol on B16F10 and CT26 cells

To confirm whether hinokitiol regulates EpCAM expression via the Akt/mTOR signaling pathway, a constitutively active Akt gene was transfected into the cells [25], and Western blotting analysis was used to observe the effects of hinokitiol on the Akt/mTOR signaling pathway and epithelial cell adhesion molecules. If the constitutively active Akt results in higher expression of downstream genes like phospho-AkT and phosoho-mTOR compared to the non-transfected group, and if hinokitiol treatment significantly reduces this expression, it can be concluded that hinokitiol inhibits the Akt/mTOR signaling pathway to regulate EpCAM expression in B16F10 and CT26 cells. Based on the findings in **Figure 3**, which showed significant inhibitory effects at the highest concentration of hinokitiol (12.5 μM), this concentration was selected for further testing. B16F10 and CT26 cells were transfected with the constitutively

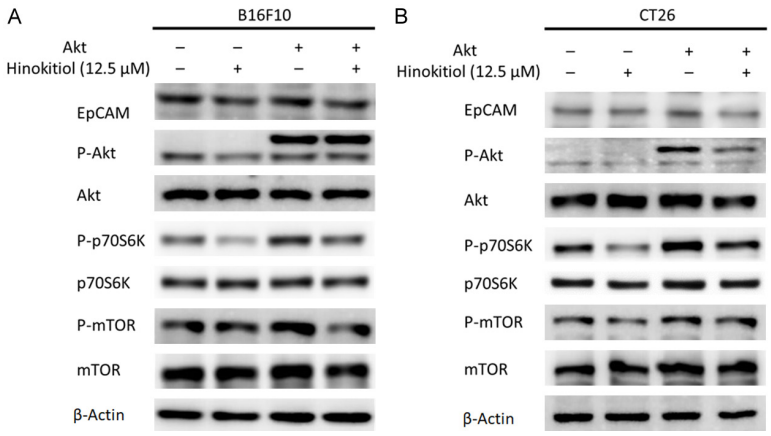

Figure 3. Constitutively active-Akt reduces the effect of hinokitiol on B16F10 and CT26 cells. (A) B16F10 and (B) CT26 cells were transfected with constitutively active AKT plasmid for 16 hours, and then treated with hinokitiol (12.5 μM) or not treated for 16 hours. Western blotting was used to detect the expression of EpCAM, P-Akt, P-mTOR and P-p70S6K proteins in B16F10 and CT26 cells. β-Actin is expressed as a loading control for total protein. Each experiment was repeated three times with similar results. *, P<0.05; **, P<0.01; ***, P<0.001 (Mean ± SD, n = 3).

active Akt plasmids, followed by hinokitiol treatment for 16 hours. **Figure 3A** and **3B** show that hinokitiol inhibited the overexpressed Akt/mTOR signaling pathway proteins and the EpCAM expression mediated by constitutively active Akt. Integrating the results from **Figures 2** and **3**, it can be concluded that hinokitiol regulates EpCAM expression through the Akt/mTOR/p70S6K signaling pathway (Figures S3 and S4).

Migration ability of B16F10 and CT26 cells after hinokitiol treatment

Given that hinokitiol inhibited EpCAM expression (**Figure 2**), we hypothesized that hinokitiol could reduce tumor cell migration. To test this, we first used a wound healing assay to observe the migration distance of B16F10 and CT26 tumor cells following treatment with 12.5 μM hinokitiol. The migration distance of B16F10 and CT26 cells was significantly reduced upon the addition of hinokitiol compared to the control groups (**Figure 2**). To ensure that the observed wound healing effect was not due to cell proliferation (**Figure 4**), we conducted a Transwell assay to examine and confirm the migration capability of the tumor cells. As expected, the migration of B16F10 and CT26 tumor cells was significantly reduced after hinokitiol treatment in the Transwell assay, as shown in **Figure 5**. These results demonstrate that hinokitiol can decrease cell mobility, and therefore, may be effective in preventing the migration process.

Transwell assays to evaluate the inhibitory effect of constitutively active-Akt and hinokitiol on tumor cell migration

In this study, hinokitiol reduced EpCAM levels in tumor cells by decreasing Akt phosphorylation. The effect on the Akt/mTOR/p70S6K signaling pathway was reversed by transfection with constitutively active Akt plasmids. Specifically, the inhibitory effect of hinokitiol on the Akt/mTOR/p70S6K signaling pathway was alleviated by transfecting B16F10 (**Figure 5A** and **5B**) and CT26 (**Figure 5A** and **5C**) cells with constitutively active AKT. Transfection with the constitutively active Akt plasmid slightly increased EpCAM expression following hinokitiol treatment compared to the control group (**Figure 3**). This increase was further enhanced by transfecting the two tumor cell lines with constitutively active Akt plasmids. Then, Transwell assay was used to assess the role of Akt in tumor cell migration. As shown in **Figure 6**, hinokitiol significantly reduced the migration of B16F10 and CT26 cells even after transfection with constitutively active Akt plasmids.

Hinokitiol prolonged the survival of tumor-bearing mice in vivo

Our findings above elucidated the physiological effects of hinokitiol *in vitro*, particularly focusing on cell migration. We further validated these results *in vivo*. B16F10 and CT26 tumor cells, pre-incubated with or without hinokitiol (control), were injected into mice via the tail vein. At day 15, the mice were sacrificed, and the tumor weight in the lungs was measured. Histological examinations of lung tissue sections revealed a greater number of tumor nodules in the mice that received only B16F10 and CT26 cells compared to those injected with cells mixed with hinokitiol (**Figure 7A**). Additionally, the weight of lung tumors-treated hinokitiol in the mice was significantly lower

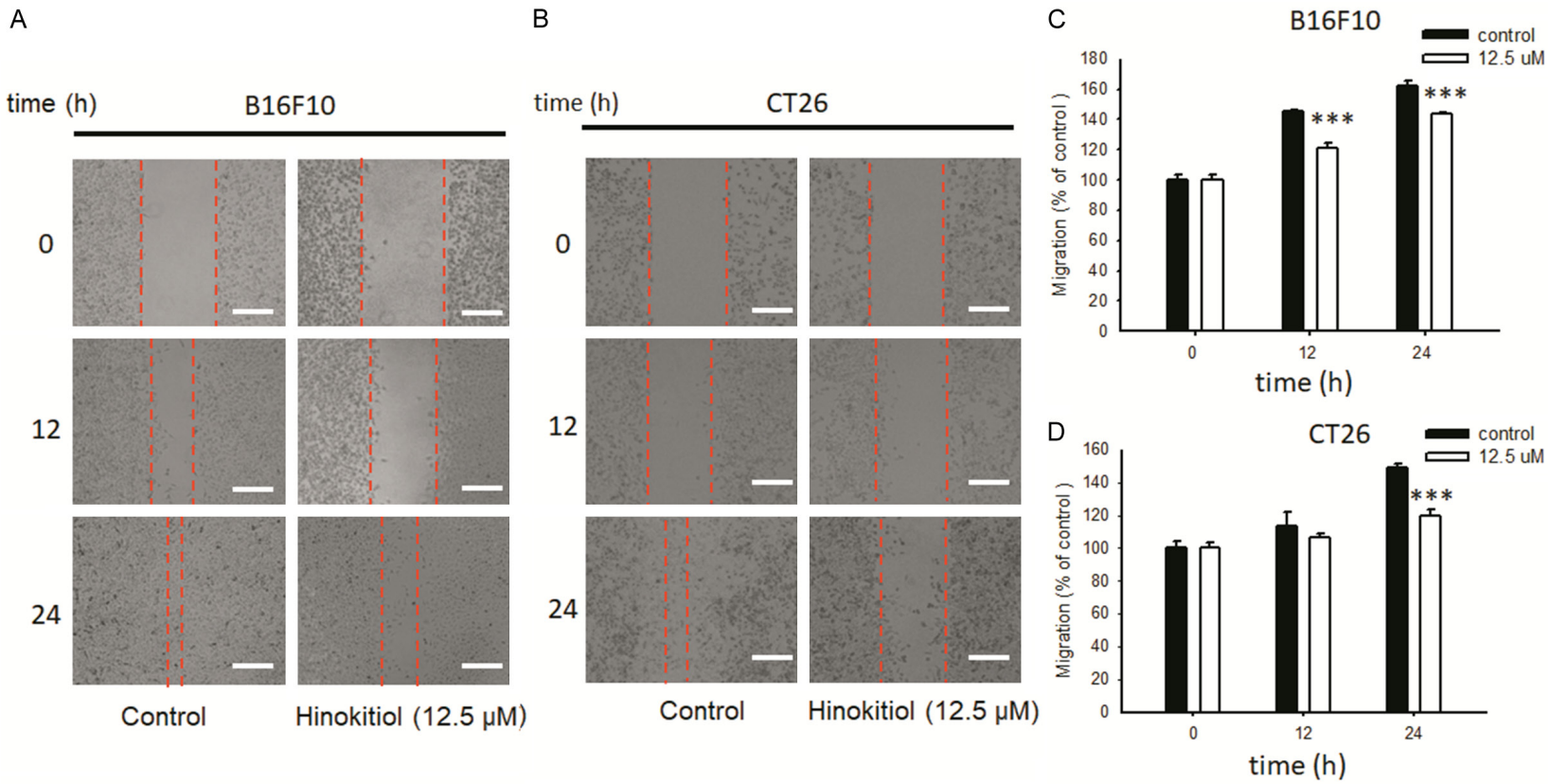

Figure 4. Cell migration ability of B16F10 and CT26 cells after treatment with hinokitiol. Wound healing assays were performed, and the cells were co-cultured with hinokitiol for 16 hours. The movement distances of (A) B16F10 and (B) CT26 cells were observed at 0, 12, and 24 hours respectively (×200) scale bar, 60 μm and the measurements are shown in (C, D).

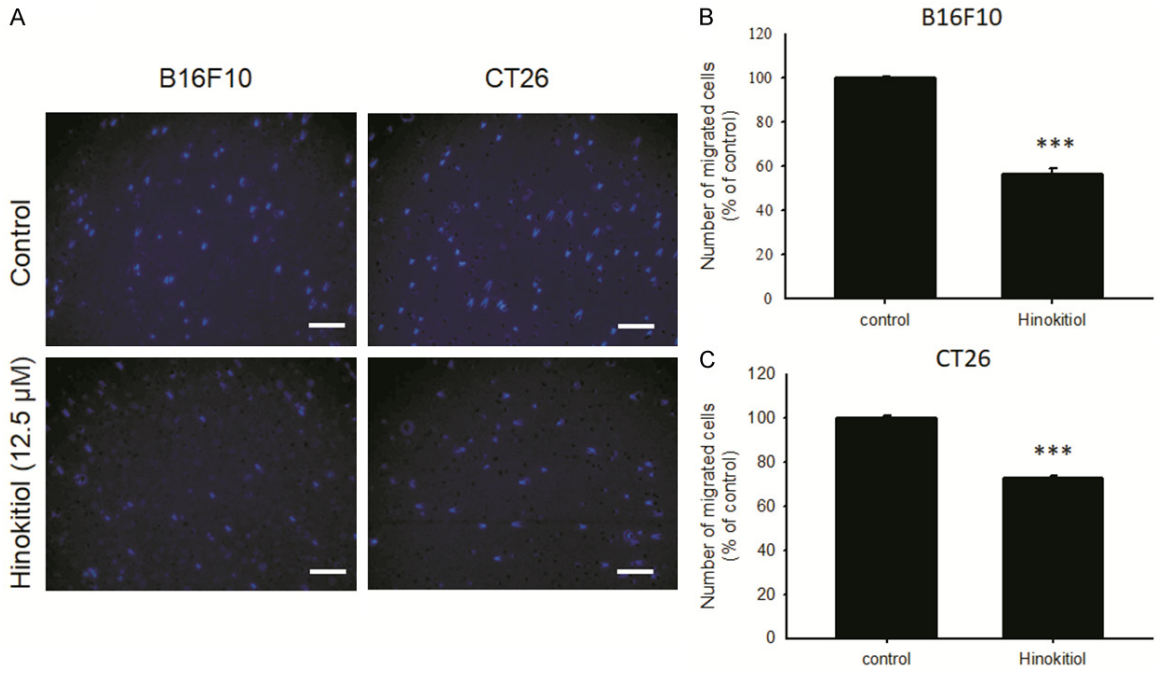

Figure 5. Effects of hinokitiol on cellular migration in B16F10 and CT26 cells. Transwell assays, (A) B16F10 and CT26 cells were treated with hinokitiol (12.5 μM) for 16 hours, and stained with DAPI in the lower layer of Tranwell. (B) B16F10 cells and (C) CT26 cells were counted under a fluorescence microscope (×400) scale bar, 30 μm. *, P<0.05; **, P<0.01; ***, P<0.001 (Mean ± SD, n = 3).

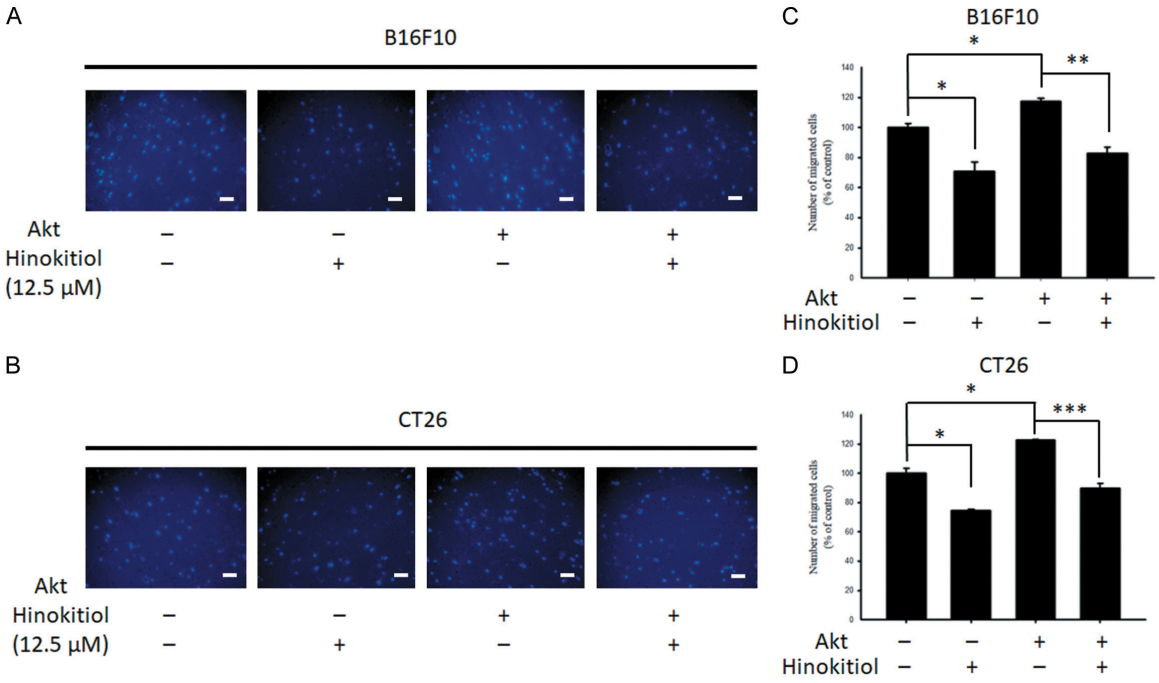

Figure 6. Transwell assays was used to detect the inhibitory effect of constitutively active-AT and hinokitiol on tumor cell migration. (A, C) B16F10 and (B, D) CT26 cells were transfected with persistently activated AKT plasmid. After 12-16 hours, B16F10 and CT26 cells were placed on the upper layer of Tranwell and then treated with hinokitiol for 16 hours. The lower layer of Tranwell was stained with 4',6-diamidino-2-phenylindole (DAPI), and (C) B16F10 cells and (D) CT26 cells were counted under a fluorescence microscope (×400) scale bar, 30 μm. *, P<0.05; **, P<0.01; ***, P<0.001 (Mean ± SD, n = 3).

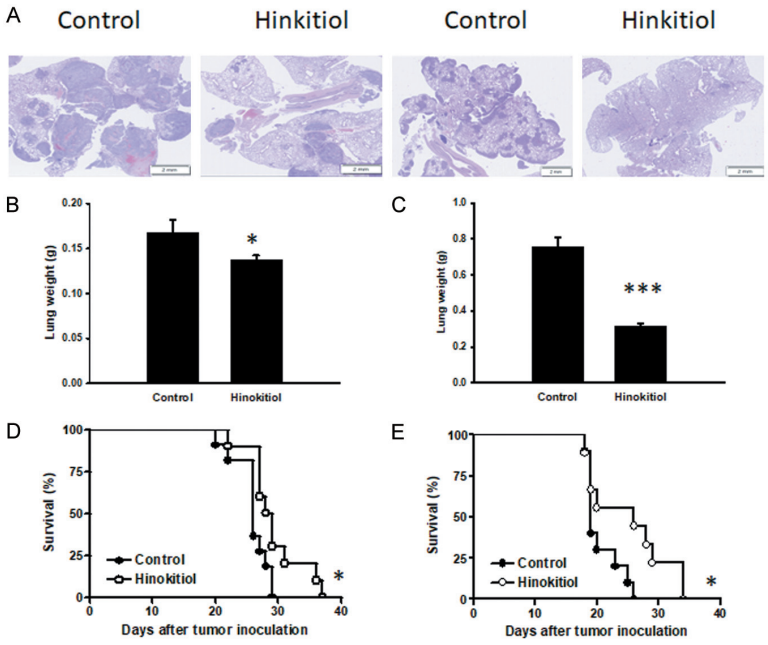

Figure 7. Hinokitiol inhibited metastasis *in vivo*. B16F10 and CT26 cells were treated with hinokitiol or not, then cells were injected into mice via tail vein. (A) Histological sections of lung tissue showing metastatic pulmonary tumor nodules observed at 15 days post-intravenous injection of cells (n = 5) (×40) scale bar, 2 mm. (B) After 15 days, mice were sacrificed and the lung tumor weight were measured (n = 3). (C) Recorded mice survival after tumor inoculation (n = 10). Recorded mice survival after tumor (D) (B16F10) and (E) (CT26) inoculation (n = 10).

than that of the PBS control group, as shown in **Figure 7B** and **7C**. Regarding the survival rate of the mice, we recorded the day each animal died and found that the lifespan was extended in the hinokitiol-treated group (**Figure 7D** and **7E**). These results demonstrate that hinokitiol effectively impedes tumor metastasis *in vivo* and prolongs the survival rate of the mice.

Discussion

Migration and invasion are hallmark behaviors of aggressive and malignant tumor cells. Despite significant advancements in cancer treatment, metastasis, chemoresistance, and tumor relapse remain substantial barriers to effective therapy. Overcoming these challenges requires strategies that disrupt the physiological activities critical for tumor growth and survival. Previous studies have shown that hinokitiol can inhibit tumor growth by inducing autophagy [26] and promoting ferroptosis [17].

In this study, we investigated the effects of hinokitiol on the highly invasive B16F10 melanoma cells and aggressive CT26 colorectal carcinoma cells. B16F10 cells are known to metastasize to the lungs following intravenous injection, while CT26 cells spontaneously metastasize to multiple sites, including lymph nodes, blood, brain, lungs, and liver. We identified EpCAM as a key target of hinokitiol in reducing tumor migration and invasion. Consistent with previous evidence linking EpCAM to these processes [3, 27], our findings demonstrate that hinokitiol treatment significantly decreased EpCAM expression in both cell lines. Reduced migration was observed in wound healing and Transwell assays, further supporting hinokitiol's anti-metastatic effects.

To elucidate the mechanism underlying this effect, we examined the Akt/mTOR signaling pathway, which is known to regulate EpCAM expression. Hinokitiol inhibited Akt and mTOR phosphorylation, resulting in reduced EpCAM expression and suppressed tumor cell migration. In contrast, constitutive activation of Akt increased EpCAM expression and promoted cell migration. However, hinokitiol treatment not only reversed Akt phosphorylation but also downregulated EpCAM expression, as confirmed by Transwell assays.

Animal studies further validated the anti-metastatic effects of hinokitiol. Tumor-bearing mice treated with hinokitiol exhibited fewer metastatic nodules, lighter lung weights, and longer survival times compared to controls. Importantly, no toxicity or carcinogenic effects were observed, highlighting hinokitiol's safety profile. These results underscore the compound's ability to inhibit metastasis *in vitro* and *in vivo* by targeting multiple pathways.

Beyond its impact on EpCAM and Akt/mTOR, hinokitiol exhibits pleiotropic activity by modulating several other pathways, including matrix metalloproteinase (MMP) suppression [28],

hypoxia-inducible factor 1-α (HIF-1α) downregulation [29], and inhibition of Wnt/β-catenin [30], NF-κB [31], and MAPK/ERK [28]. These multifaceted effects emphasize hinokitiol's potential as a versatile therapeutic agent with anti-inflammatory, anti-metastatic, and pro-apoptotic properties.

In conclusion, our study highlights EpCAM as a critical target of hinokitiol, underscoring its role as a potent anticancer candidate. By inhibiting the Akt/mTOR pathway, hinokitiol downregulates EpCAM expression, reducing tumor migration and metastasis in vitro and in vivo. These findings pave the way for further investigation into hinokitiol's mechanisms of action and potential side effects, with the ultimate goal of developing more effective and targeted cancer therapies. Hinokitiol-mediated inhibition of tumor-induced EpCAM expression offers a promising approach for suppressing tumor growth and metastasis, representing a significant step forward in cancer treatment strategies.

Acknowledgements

This work was supported by Higher Education Sprout Project and Kaohsiung Armed Forces General Hospital, Taiwan.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Che-Hsin Lee, Department of Biological Sciences, National Sun Yat-sen University, No. 70 Lienhai Road, Kaohsiung 80424, Taiwan. E-mail: chlee@mail.nsysu.edu.tw

References

[1] Steeg PS. Tumor metastasis: mechanistic insights and clinical challenges. Nat Med 2006; 12: 895-904.

[2] Pangilinan CR, Wu LH and Lee CH. *Salmonella* impacts tumor-induced macrophage polarization, and inhibits SNAI1-mediated metastasis in melanoma. Cancers (Basel) 2021; 13: 2894.

[3] Yen WC, Li QZ, Wu LH, Lee WY, Chang WW, Chien PJ and Lee CH. *Salmonella* inhibits tumor metastasis by downregulating epithelial cell adhesion molecules through the protein kinase-B/mammalian target of rapamycin signaling pathway. Int J Biol Macromol 2023; 253: 126913.

[4] Munz M, Baeuerle PA and Gires O. The emerging role of EpCAM in cancer and stem cell signaling. Cancer Res 2009; 69: 5627-5629.

[5] Mohtar MA, Syafruddin SE, Nasir SN and Low TY. Revisiting the roles of pro-metastatic EpCAM in cancer. Biomolecules 2020; 10: 255.

[6] Panda SS, Lee CC, Geevimaan K, Chen KC, Yang SH, Shen CN, HuangFu WC and Wu HC. Intracellular domain of epithelial cell adhesion molecule induces Wnt receptor transcription to promote colorectal cancer progression. J Biomed Sci 2024; 31: 72.

[7] Mulè P, Fernandez-Perez D, Amato S, Manganaro D, Oldani P, Brandini S, Diaferia G, Cuomo A, Recordati C, Soriani C, Dondi A, Zanotti M, Rustichelli S, Bisso A, Pece S, Rodighiero S, Natoli G, Amati B, Ferrari KJ, Chiacchiera F and Pasini D. WNT oncogenic transcription requires MYC suppression of lysosomal activity and EPCAM stabilization in gastric tumors. Gastroenterology 2024; 167: 903-918.

[8] Xiao D, Xiong M, Wang X, Lyu M, Sun H, Cui Y, Chen C, Jiang Z and Sun F. Regulation of the function and expression of EpCAM. Biomedicines 2024; 12: 1129.

[9] Winter MJ, Nagelkerken B, Mertens AE, Rees-Bakker HA, Briaire-de Bruijn IH and Litvinov SV. Expression of Ep-CAM shifts the state of cadherin-mediated adhesions from strong to weak. Exp Cell Res 2003; 285: 50-58.

[10] Chen HN, Liang KH, Lai JK, Lan CH, Liao MY, Hung SH, Chuang YT, Chen KC, Tsuei WW and Wu HC. EpCAM signaling promotes tumor progression and protein stability of PD-L1 through the EGFR pathway. Cancer Res 2020; 80: 5035-5050.

[11] Ni YJ, Huang ZN, Li HY, Lee CC, Tyan YC, Yang MH, Pangilinan CR, Wu LH, Chiang YC and Lee CH. Hinokitiol impedes tumor drug resistance by suppressing protein kinase B/mammalian targets of rapamycin axis. J Cancer 2022; 13: 1725-1733.

[12] Liu YH, Wu LH, Fan WJ, Chiu SH, Chen PH, Wang CC and Lee CH. A tellurium-based small compound ameliorates tumor metastasis by downregulating heparanase expression. J Cancer 2024; 15: 5308-5317.

[13] Dehelean CA, Marcovici I, Soica C, Mioc M, Coricovac D, Iurciuc S, Cretu OM and Pinzaru I. Plant-derived anticancer compounds as new perspectives in drug discovery and alternative therapy. Molecules 2021; 26: 1109.

[14] Zhao M, Qiao C, Yang S, Tang Y, Sun W, Sun S, Guo Q, Du F, Zhang N, Ning T, Wu J, Xu J and Li P. Hinokitiol protects gastric injury from ethanol exposure via its iron sequestration capacity. Eur J Pharmacol 2024; 966: 176340.

[15] Tai LR, Chiang YF, Huang KC, Chen HY, Ali M and Hsia SM. Hinokitiol as a modulator of TLR4 signaling and apoptotic pathways in atopic dermatitis. Biomed Pharmacother 2024; 170: 116026.
[16] Tang H, He K, Zhao K, Zheng C, Wu W, Jin W, Yang L and Xie B. Protective effects of hinokitiol on neuronal ferroptosis by activating the Keap1/Nrf2/HO-1 pathway in traumatic brain injury. J Neurotrauma 2024; 41: 734-750.
[17] Zhao H, Zhang M, Zhang J, Sun Z, Zhang W, Dong W, Cheng C, Yao Y and Li K. Hinokitiol-iron complex is a ferroptosis inducer to inhibit triple-negative breast tumor growth. Cell Biosci 2023; 13: 87.
[18] Qin M, Shao B, Lin L, Zhang ZQ, Sheng ZG, Qin L, Shao J and Zhu BZ. Molecular mechanism of the unusual biphasic effects of the natural compound hinokitiol on iron-induced cellular DNA damage. Free Radic Biol Med 2023; 194: 163-171.
[19] Wu LH, Huang YT, Lin CY and Lee CH. *Salmonella*-induced inhibition of β3-adrenoceptor expression in tumors and reduces tumor metastasis. J Cancer 2024; 15: 1203-1212.
[20] Lin CY, Wu CY, Wang CC and Lee CH. Exposure to phenols reduces melanogenesis in B16F10 cells and zebrafish. Aquat Toxicol 2024; 266: 106806.
[21] Chiu HM, Chiou WY, Hsu WJ, Wu LH, Yang MH, Tyan YC and Lee CH. *Salmonella* alters heparanase expression and reduces tumor metastasis. Int J Med Sci 2021; 18: 2981-2989.
[22] Lee TH, Lin GY, Yang MH, Tyan YC and Lee CH. *Salmonella* reduces tumor metastasis by downregulation C-X-C chemokine receptor type 4. Int J Med Sci 2021; 18: 2835-2841.
[23] Wu YJ, Hsu WJ, Wu LH, Liou HP, Pangilinan CR, Tyan YC and Lee CH. Hinokitiol reduces tumor metastasis by inhibiting heparanase via extracellular signal-regulated kinase and protein kinase B pathway. Int J Med Sci 2020; 17: 403-413.
[24] Tsao YT, Huang YF, Kuo CY, Lin YC, Chiang WC, Wang WK, Hsu CW and Lee CH. Hinokitiol inhibits melanogenesis via AKT/mTOR signaling in B16F10 mouse melanoma cells. Int J Mol Sci 2016; 17: 248.
[25] Yang CJ, Chang WW, Lin ST, Chen MC and Lee CH. *Salmonella* overcomes drug resistance in tumor through P-glycoprotein downregulation. Int J Med Sci 2018; 15: 574-579.
[26] Wang WK, Lin ST, Chang WW, Liu LW, Li TY, Kuo CY, Hsieh JL and Lee CH. Hinokitiol induces autophagy in murine breast and colorectal cancer cells. Environ Toxicol 2016; 31: 77-84.
[27] Kanahori M, Shimada E, Matsumoto Y, Endo M, Fujiwara T, Nabeshima A, Hirose T, Kawaguchi K, Oyama R, Oda Y and Nakashima Y. Immune evasion in lung metastasis of leiomyosarcoma: upregulation of EPCAM inhibits CD8(+) T cell infiltration. Br J Cancer 2024; 130: 1083-1095.
[28] Huang CH, Lu SH, Chang CC, Thomas PA, Jayakumar T and Sheu JR. Hinokitiol, a tropolone derivative, inhibits mouse melanoma (B16-F10) cell migration and in vivo tumor formation. Eur J Pharmacol 2015; 746: 148-157.
[29] Lee MJ, Kim JW and Yang EG. Hinokitiol activates the hypoxia-inducible factor (HIF) pathway through inhibition of HIF hydroxylases. Biochem Biophys Res Commun 2010; 396: 370-375.
[30] Li J, Zhou XD, Yang KH, Fan TD, Chen WP, Jiang LF, Bao JP, Wu LD and Xiong Y. Hinokitiol reduces matrix metalloproteinase expression by inhibiting Wnt/beta-Catenin signaling in vitro and in vivo. Int Immunopharmacol 2014; 23: 85-91.
[31] Chelpuri Y, Pabbathi S, Alla GR, Yadala RK, Kamishetti M, Banothu AK, Boinepally R, Bharani KK and Khurana A. Tropolone derivative hinokitiol ameliorates cerulein-induced acute pancreatitis in mice. Int Immunopharmacol 2022; 109: 108915.

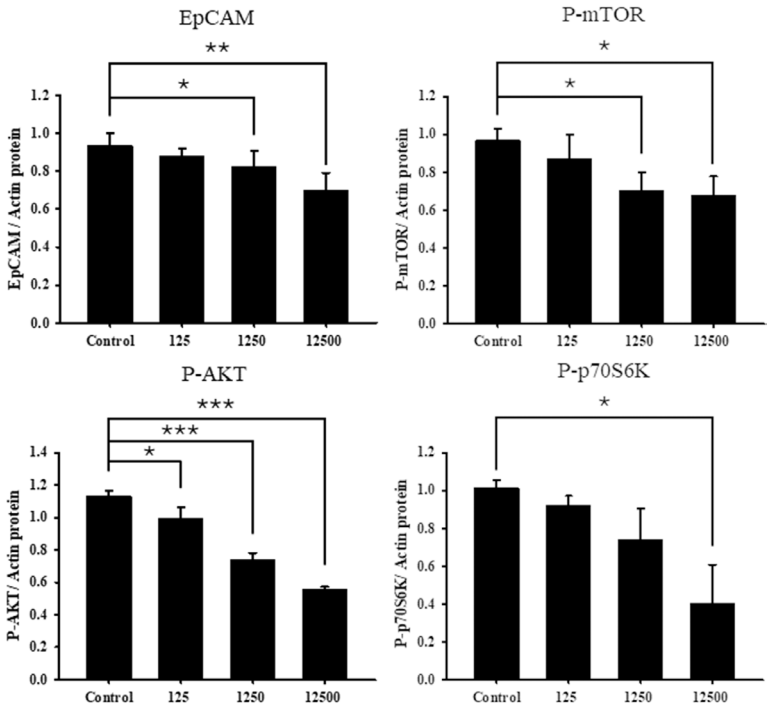

Figure S1. The EpCAM/P-Akt/P-mTOR/P-p70S6K phosphorylation expression levels following various concentration hinokitiol treatment in B16F10 cells. Quantified band intensities normalized to β-Actin (n = 3, mean ± SD. *P<0.05; ***P<0.001).

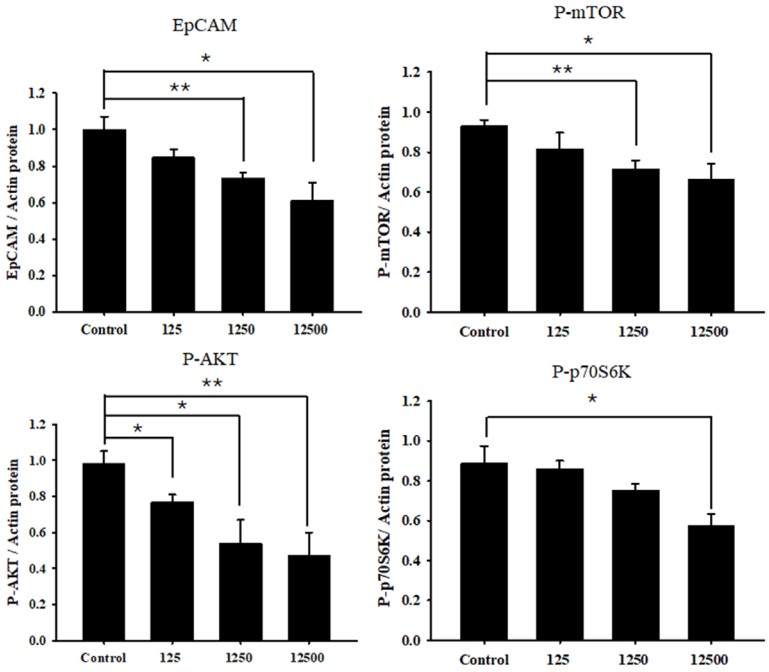

Figure S2. The EpCAM/P-Akt/P-mTOR/P-p70S6K phosphorylation expression levels following various concentration hinokitiol treatment in CT26 cells. Quantified band intensities normalized to β-Actin (n = 3, mean ± SD. *P<0.05; **P<0.01; ***P<0.001).

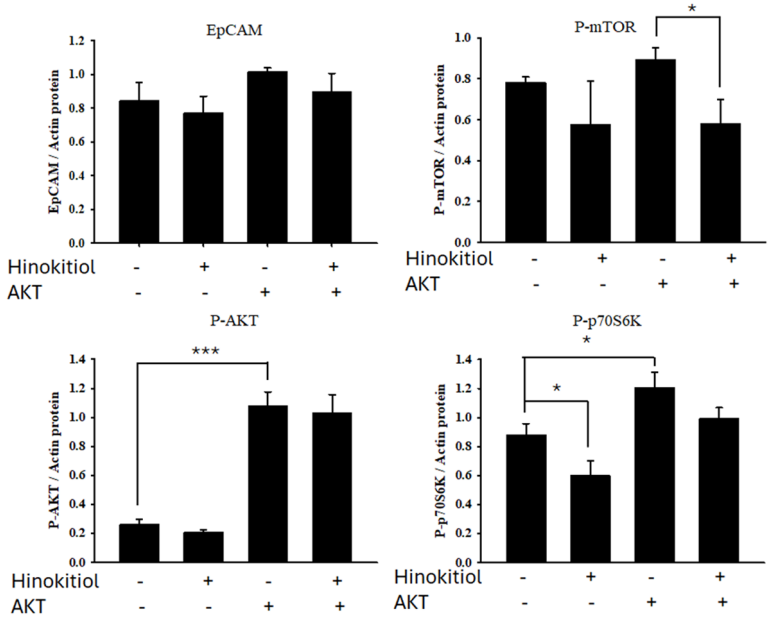

Figure S3. B16F10 cells were transfected with constitutively active Akt plasmid for 16 hours, and then treated with hinokitiol (12.5 μM) or not treated for 16 hours. The EpCAM/P-Akt/P-mTOR/P-p70S6K phosphorylation expression levels. Quantified band intensities normalized to β-Actin (n = 3, mean ± SD. *P<0.05; **P<0.01; ***P<0.001).

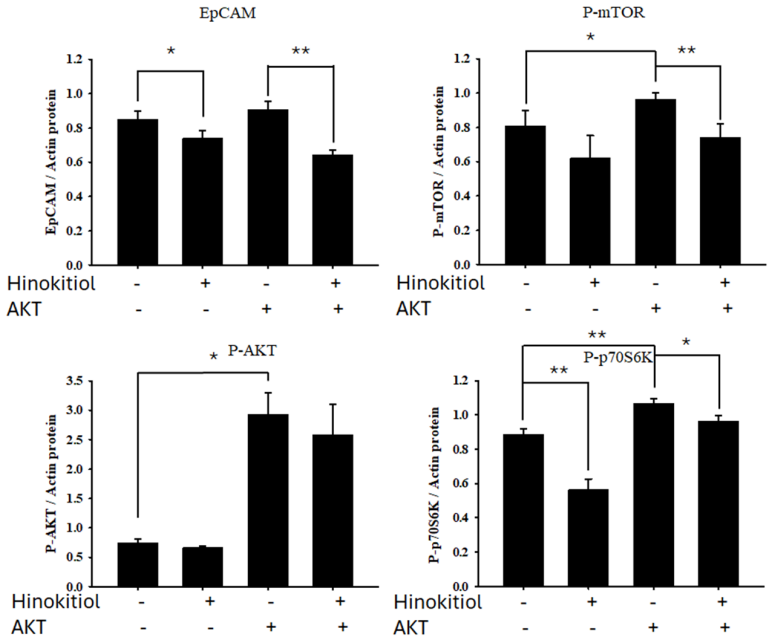

Figure S4. CT26 cells were transfected with constitutively active Akt plasmid for 16 hours, and then treated with hinokitiol (12.5 μM) or not treated for 16 hours. The EpCAM/P-Akt/P-mTOR/P-p70S6K phosphorylation expression levels. Quantified band intensities normalized to β-Actin (n = 3, mean ± SD. *P<0.05; **P<0.01).