Swainsonine Inhibits Invasion and the EMT Process in Esophageal Carcinoma Cells by Targeting Twist1

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INTRODUCTION

Esophageal cancer is a common gastrointestinal cancer and is most prevalent in Asia (including China and Central Asia) and in East and South Africa. In 2012, about 455,800 new cases of esophageal cancer were recorded worldwide, about 400,200 people died from esophageal cancer, and more than 80% of these deaths occurred in developing countries. Esophageal cancer is generally three to four times more common among males than females. Esophageal cancer shows no signs or symptoms in the early stage; it is usually diagnosed at an advanced stage, with a 5-year survival rate of 12%. Dysphagia and weight loss are the most common presenting features at advanced stage. Treatment options include surgery, radiotherapy, chemotherapy, targeted therapy, and endoscopic interventions. Despite the available treatment options, patients usually die due to relapse or metastasis.

Swainsonine is a cytoxic fungal alkaloid, produced by fungal symbionts of plants (endophytes), plant pathogens, or insect pathogens. Swainsonine primarily inhibits α-mannosidase II and lyosyme α-mannosidase activities. Swainsonine takes part in a wide range of biological functions, including weight loss, decreased libido, altered behavior, depression, abortion, infertility, birth defects, and death. Swainsonine disrupts the endomembrane system of cells by inhibiting α-mannosidase II and is therefore under consideration as a chemotherapeutic treatment option for cancer. Santos et al. showed that the administration of swainsonine in combination with cisplatin increases the sensitivity of Ehrlich ascites carcinoma cells to cisplatin. Swainsonine has also been reported to inhibit cell growth of lung cancer cells and esophageal squamous carcinoma cells via a mitochondria-mediated, caspase-dependent apoptotic pathway both in vitro and in vivo. In the present study, we further explored the effects of swainsonine on esophagus cancer, with more focus on cell invasion and metastasis, as the survival rate is much less (4%) in patients with metastasis.
was then maintained at 37°C in an atmosphere of 5% CO₂–95% air. Transforming growth factor-β1 (TGF-β1; 10 ng/ml) was used to induce epithelial–mesenchymal transition (EMT) in the cells. Swainsonine (Fig. 1) was purchased from Sigma-Aldrich (S6640; St. Louis, MO, USA) and was dissolved in dimethyl sulfoxide (DMSO). Swainsonine was used to treat cells at 10, 20, 50, and 100 µg/ml concentrations in the following experiments.

**CCK-8 Assay**

Cell proliferation was assessed by cell counting kit-8 (CCK-8; Dojindo Molecular Technologies, Gaithersburg, MD, USA). Cells were seeded in a 96-well plate with 5,000 cells/well. After stimulation, the CCK-8 solution was added to the culture medium, and the cultures were incubated for 1 h at 37°C in humidified 95% air and 5% CO₂. The absorbance was measured at 450 nm using a Microplate Reader (Bio-Rad, Hercules, CA, USA).

**Cell Invasion Assay**

Cell invasion was measured using 24-well Millicell Hanging Cell Culture Inserts with 8-µm polyethylene terephthalate (PET) membranes (Millipore, Bedford, MA, USA). Briefly, after the cells were treated for indicated conditions, 5.0×10⁴ cells in 200 µl of serum-free Dulbecco’s modified Eagle’s medium (DMEM) were plated onto BD BioCoat™ Matrigel™ Invasion Chambers (8-µm pore size polycarbonate filters; BD Biosciences, San Jose, CA, USA), while complete medium containing 10% FBS was added to the lower chamber. After processing the invasion chambers for 48 h (37°C, 5% CO₂) in accordance with the manufacturer’s protocol, the noninvading cells were removed with a cotton swab; the invading cells were fixed in 100% methanol and then stained with crystal violet solution and counted microscopically. The data were presented as average number of cells attached to the bottom surface from five randomly chosen fields.

**Apoptosis Assay**

Cell apoptosis was measured using propidium iodide (PI) and fluorescein isothiocynate (FITC)-conjugated annexin V staining. Briefly, cells were washed in phosphate-buffered saline (PBS). These cells were then washed twice in PBS and stained in PI/FITC-annexin V in the presence of 50 µg/ml RNase A (Sigma-Aldrich), and then incubated for 1 h at room temperature in the dark. Flow cytometry analysis was done using a FACSScan (Beckman Coulter, Fullerton, CA, USA). The data were analyzed using FlowJo software.

**Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)**

Total RNA was extracted from cells using TRIzol reagent (Life Technologies Corporation, Carlsbad, CA, USA) according to the manufacturer’s instructions. One Step SYBR® PrimeScript® PLUS Kit (TaKaRa Biotechnology, Dalian, P.R. China) was used for qRT-PCR analysis to measure the expression levels of Twist1 in the cells.

**Western Blot**

The proteins used for Western blotting were extracted using RIPA lysis buffer (Beyotime Biotechnology, Shanghai, P.R. China) supplemented with protease inhibitors (Roche, Guangzhou, P.R. China). The proteins were quantified using BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). Western blot system was established using a Bio-Rad Bis-Tris Gel system according to the manufacturer’s instructions. Primary antibodies were prepared in 5% blocking buffer at a dilution of 1:1,000. Primary antibodies were incubated with the membrane at 4°C overnight, followed by washing and incubation with secondary antibody tagged by horseradish peroxidase for 1 h at room temperature. After rinsing, the polyvinylidene difluoride blot membrane and antibodies were transferred into the Bio-Rad ChemiDoc™ XRS system, and then 200 µl of Immobilon Western Chemiluminescent HRP Substrate (Millipore) was added to cover the membrane surface. The signals were captured, and the intensity of the bands was quantified using Image Lab™ Software (Bio-Rad, Shanghai, P.R. China). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control.

**Statistical Analysis**

All experiments were repeated three times. The results of multiple experiments are presented as mean±standard deviation. Statistical analyses were performed using SPSS 19.0 statistical software. Values of p were calculated using a one-way analysis of variance. A value of p<0.05 was considered to indicate a statistically significant result.

**RESULTS**

**Effects of Swainsonine on Cell Viability, Apoptosis, and Invasion**

Human esophageal carcinoma cells were treated with different concentrations of swainsonine (10, 20, 50, and 100 µg/ml) for 24 hours.
100 µg/ml); nontreated cells served as control. Cell viability was measured using the CCK-8 assay, apoptosis using flow cytometry, and invasion using Transwell invasion assay. Results showed that swainsonine had no effect on cell viability (Fig. 2A) and apoptosis (Fig. 2B), but it significantly decreased cell invasion at 20, 50, and 100 µg/ml concentrations compared with that of the control cells (p<0.05) (Fig. 2C). We measured cell viability and apoptosis using Western blot. Results showed that swainsonine had no effect on the expressions of

![Figure 2](image-url)

**Figure 2.** Effects of swainsonine on cell viability, apoptosis, and invasion. Esophageal carcinoma cells were treated with swainsonine at different concentrations (10, 20, 50, and 100 µg/ml); nontreated cells served as control. (A) Cell viability was measured using cell counting kit-8 (CCK-8) assay. (B) Cell apoptosis was measured using flow cytometry. (C) Cell invasion was measured using Transwell invasion assay. (D) Western blot was used to measure the expressions of viability-associated proteins [proliferating cell nuclear antigen (PCNA), cyclin A, cyclin E1, cyclin-dependent kinase 2 (CDK2), cyclin D1, and CDK4]. (E) Western blot was used to measure the expressions of apoptosis-related proteins [B-cell lymphoma 2 (Bcl-2), Bcl-2 X-associated protein (Bax), cleaved caspase 3, and cleaved caspase 9]. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. *p<0.05 compared to control.
viability-associated proteins [proliferating cell nuclear antigen (PCNA), cyclin A, cyclin E1, cyclin-dependent kinase 2 (CDK2), cyclin D1, and CDK4] (Fig. 2D) as well as on the expressions of apoptosis-related proteins [B-cell lymphoma 2 (Bcl-2), Bcl-2 X-associated protein (Bax), cleaved caspase 3, and cleaved caspase 9] (Fig. 2E). These results indicate that swainsonine inhibits invasion of esophageal carcinoma cells but does not affect cell viability and apoptosis.

**Effects of Swainsonine on EMT**

Because swainsonine inhibited cell invasion, we measured its effect on EMT using Western blot. Cells were treated with TGF-β1 (10 ng/ml) to induce EMT in the cells. Swainsonine was used at different concentrations (10, 20, 50, and 100 µg/ml); nontreated cells served as control. As shown in Figure 3, swainsonine increased the expression of E-cadherin but decreased the expression of N-cadherin, vimentin, zinc finger E-box-binding homeobox 1 (ZEB1), and snail in a dose-dependent manner compared to the expressions of the same proteins in the control TGF-β1-treated cells. These results indicate that swainsonine inhibits EMT in esophageal carcinoma cells.

**Effects of Swainsonine on Twist1**

We then measured the relative mRNA expression of Twist1 in esophageal carcinoma cells using qRT-PCR. Cells were treated with swainsonine at different concentrations (10, 20, 50, and 100 µg/ml); nontreated cells served as control. As shown in Figure 4A, swainsonine significantly decreased the mRNA expression of Twist1 in the cells at 50 and 100 µg/ml concentrations compared with the control (all \( p < 0.05 \)). Similar results were found in the Western blot analysis (Fig. 4B).

**Swainsonine Inhibits Cell Invasion and EMT by Downregulation of Twist1 and Deactivation of the PI3K/AKT Signaling Pathway**

Esophageal carcinoma cells were transfected with pEX or pEX-Twist1; nontreated cells served as control. We measured mRNA and protein expression of Twist1 in these cells using qRT-PCR and Western blot, respectively. Results showed that pEX-Twist1 significantly increased the mRNA expression (\( p < 0.01 \)) (Fig. 5A) and protein expression (Fig. 5B) of Twist1 in the cells.

For further experiments, cells were treated with TGF-β1, TGF-β1 + swainsonine (50 µg/ml), TGF-β1 + pEX + swainsonine (50 µg/ml), or TGF-β1 + pEX-Twist1 + swainsonine (50 µg/ml). Nontreated cells served as control. We then measured cell invasion using Transwell invasion filters.
Swainsonine inhibits cell invasion and EMT by downregulation of Twist1 and deactivation of the phosphoinositide 3-kinase (PI3K)/AKT signaling pathway. Esophageal carcinoma cells were transfected with pEX or pEX-Twist1; untransfected cells served as control. The mRNA and protein expressions of Twist1 were measured using qRT-PCR (A) and Western blot (B), respectively. Then cells were treated with TGF-β1, TGF-β1 + swainsonine (50 µg/ml), TGF-β1 + pEX + swainsonine (50 µg/ml), or TGF-β1 + pEX-Twist1 + swainsonine (50 µg/ml), and the following experiments were carried out: (C) Transwell invasion assay, to measure cell invasion; and Western blot, to measure the expression of (D) E-cadherin, N-cadherin, vimentin, ZEB1, and snail, and (E) PI3K and AKT. *p<0.05, **p<0.01 as indicated.
swainsonine inhibited EMT in the cells by downregulation of Twist1 (Fig. 5D). We also measured the expression of the phosphoinositide 3-kinase (PI3K)/AKT signaling pathway proteins in these cells using Western blot. As shown in Figure 5E, TGF-β1 increased the expression of PI3K and AKT, but the addition of swainsonine decreased these expressions, whereas the addition of pEX-Twist1 again increased these expressions.

These findings together indicate that swainsonine inhibits cell invasion and EMT in esophageal carcinoma cells by downregulation of Twist1 and deactivation of the PI3K/AKT signaling pathway.

DISCUSSION

In in vitro and in vivo studies, swainsonine has shown inhibitory and antimetastatic effects on several human and mouse cancer cell lines21–25. These studies provided the rationale for exploring swainsonine as an antitumor agent. In the present study, we evaluated the effects of swainsonine on human esophageal carcinoma cell lines EC9706 and 293T in vitro and found that swainsonine inhibits invasion of these cells at 20, 50, and 100 µg/ml concentrations, with maximal effect at 50 µg/ml. Similar effects were reported by Seftor et al., who showed that swainsonine, in combination with deoxymanojirimycin, inhibits human melanoma cell invasion in vitro26. Additionally, in a phase 1 study in patients with advanced malignancies, swainsonine reduced tumor cell invasion27. Apart from its effect on cell invasion, swainsonine has also been shown to decrease cancer cell growth and induce apoptosis via the mitochondrial pathway in lung cancer and esophageal cancer cells19,27. However, in our study, swainsonine had no effect on cell viability and apoptosis. This could be due to a difference in the types of cells used in these studies.

Cell invasion is followed by cell metastasis. Clinically detectable metastasis is the end product of a complex series of cellular–biological events. During metastatic progression, tumor cells exit the primary site of growth, translocate systemically, and adapt to survive and grow in distant tissues28. Metastasis is an important step in tumor progression and is a main cause of mortality in cancer patients29. After metastasis of esophageal cells to organs or lymph nodes away from the tumor, the 5-year survival rate is only 4%20.

EMT is a crucial process during which epithelial cells acquire mesenchymal properties and show reduced intercellular adhesion and increased motility. Growing evidence suggests that EMT plays an important role during tumor progression and malignant transformation, thereby providing invasive and metastatic properties to the embryonic cancer cells29. The main characteristic of EMT is loss of epithelial surface markers, most notably E-cadherin, and the acquisition of mesenchymal markers, including N-cadherin and vimentin. E-cadherin is downregulated by EMT, which is mediated by its transcriptional repression through the binding of EMT transcription factors, such as snail, Slug, and Twist, present in the E-cadherin promoter30. In cancer, E-cadherin plays a tumor suppressor role, inhibiting invasion and metastasis31. Therefore, we measured the effect of swainsonine on the expression of Twist1 and found that swainsonine significantly decreased the expression of Twist1 in esophageal carcinoma cells. In further experiments, we showed that swainsonine inhibits cell invasion and EMT in esophageal carcinoma cells by downregulation of Twist1 and deactivation of the PI3K/AKT signaling pathway. The PI3K/AKT signal pathway is an important pathway for cell survival, proliferation, and migration32. It has been reported that the use of PI3K/AKT inhibitors in combination with chemotherapy may be a potential therapeutic strategy in treating esophageal cancer33.

To the best of our knowledge, the present study is the first to explore the effects of swainsonine on cell invasion and metastasis in esophageal carcinoma cells. In summary, swainsonine decreased esophageal carcinoma cell invasion, but did not affect cell viability and apoptosis; swainsonine decreased the EMT process in the cancer cells by increasing the expression of E-cadherin and decreasing the expression of N-cadherin, vimentin, ZEB1, and snail; swainsonine also decreased the expression of Twist1 and deactivated the PI3K/AKT signaling pathway. Our findings indicate the potential inhibitory role of swainsonine in esophageal carcinoma metastasis.

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