Postprint: Molecular Mechanism of Elephantopus scaber Compound EM-12-Induced G1/S Phase Arrest and Apoptosis in 2774-C10 Cells

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Abstract

Objective: To investigate the molecular mechanism of the anti-tumor effect of ethanol extract EM-12 from Elephantopus mollis H.B.K. Methods: MTT assay was used to examine the effect of EM-12 on ovarian cancer cell viability; colony formation inhibition assay was used to assess the effect of EM-12 on the proliferative ability of ovarian cancer cells; GSEA (Gene Set Enrichment Analysis) was performed to analyze the effect of EM-12 on ovarian cancer 2774-C10 cells; PI single staining was used to detect cell cycle distribution; Annexin V-FITC/PI double staining was used to detect apoptosis; Western blotting was used to detect apoptosis- and cycle-related proteins. Results: MTT assay results demonstrated that EM-12 significantly inhibited the viability of ovarian cancer cells; RNA-seq and GSEA analysis indicated that EM-12 induced G1/S phase arrest in 2774-C10 cells; colony formation inhibition assay results showed that EM-12 significantly inhibited the proliferation of ovarian cancer cells; flow cytometry results showed that with increasing drug concentration, the apoptosis rate gradually increased, and the proportion of G1 phase cells gradually increased while the proportion of S phase cells decreased, indicating G1/S phase arrest. Western blotting results showed that with increasing drug concentration, the protein levels of cyclin E2, Cyclin D1, CDK2, and CDK6 decreased, accompanied by activation of caspase-8 and Caspase-3 and cleavage-mediated inactivation of poly ADP-ribose polymerase PARP. Conclusion: EM-12 induces G1/S phase arrest in ovarian cancer cells and induces apoptosis through the death receptor pathway.

Full Text

Molecular Mechanism of EM-12 from Elephantopus mollis H.B.K. in Inducing G1/S Phase Arrest and Apoptosis in 2774-C10 Cells

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Abstract

Objective: To investigate the molecular mechanisms underlying the antitumor effects of EM-12, an ethanol extract from Elephantopus mollis H.B.K., in ovarian cancer cells.

Methods: The effects of EM-12 on ovarian cancer cell viability were assessed using MTT assays, while colony formation assays evaluated its impact on proliferative capacity. Gene set enrichment analysis (GSEA) was performed to identify pathways affected by EM-12 in 2774-C10 ovarian cancer cells. Cell cycle distribution was analyzed by propidium iodide (PI) single staining, and apoptosis was detected via Annexin V-FITC/PI double staining. Western blotting was employed to measure the expression levels of apoptosis- and cell cycle-related proteins.

Results: MTT assays demonstrated that EM-12 significantly inhibited the viability of ovarian cancer cells in a dose-dependent manner. RNA-seq and GSEA analysis revealed that EM-12 induced G1/S phase arrest in 2774-C10 cells. Colony formation assays confirmed that EM-12 markedly suppressed the proliferative ability of ovarian cancer cells. Flow cytometry showed that apoptosis rates increased progressively with drug concentration, accompanied by a gradual increase in the proportion of G1-phase cells and a corresponding decrease in S-phase cells, indicating G1/S phase arrest. Western blotting results showed that cyclin E2, cyclin D1, CDK2, and CDK6 protein levels decreased with increasing drug concentrations, while activation of caspase-8 and caspase-3 and cleavage-mediated inactivation of poly(ADP-ribose) polymerase (PARP) were observed.

Conclusion: EM-12 induces G1/S phase arrest in ovarian cancer cells and triggers apoptosis through the death receptor pathway.

Keywords: Elephantopus mollis H.B.K., ovarian cancer, apoptosis, death receptor pathway, G1/S phase arrest

Introduction

Ovarian cancer represents the most common malignant tumor of the female reproductive system, with the highest mortality rate among gynecological malignancies. Recurrence and metastasis severely impact patient prognosis, resulting in a five-year survival rate of only 35-38%. While surgery remains the primary treatment modality, supplemented by radiotherapy and chemotherapy, the development of drug resistance—particularly to first-line chemotherapeutic agents such as paclitaxel and cisplatin—poses a significant clinical challenge. Many patients who initially respond well to paclitaxel eventually develop resistance upon tumor recurrence, drastically reducing survival rates. Therefore, identifying novel antitumor agents is of critical clinical importance for ovarian cancer therapy.

Elephantopus mollis H.B.K., a member of the Asteraceae family, has been traditionally used in Chinese medicine to treat conditions including colds, tonsillitis, hepatitis, diarrhea, and gastric diseases. The plant contains diverse chemical constituents, with sesquiterpene lactones identified as its primary bioactive components. Our research group has previously reported that EM-3, a sesquiterpene lactone monomer from this plant, induces apoptosis and G2/M phase arrest in nasopharyngeal carcinoma cells via the STAT3 pathway while reducing the proportion of side population cells. Additionally, Shao et al. demonstrated that another sesquiterpene lactone, EM-23, mediates cervical cancer cell apoptosis by targeting thioredoxin to activate JNK and cell death pathways.

In the present study, we screened 25 monomeric compounds isolated from Elephantopus mollis to identify potent antitumor agents, selecting EM-3, EM-12, and EM-23 for their pronounced activity. Since the mechanisms of EM-3 and EM-23 have been previously reported, we focused on EM-12 as our candidate compound (chemical structure shown in [Figure 1: see original paper]). This study aims to elucidate the antitumor mechanism of EM-12 in human ovarian cancer 2774-C10 cells and provide experimental evidence for its potential progression to animal and clinical studies.

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Materials and Methods

1.1 Materials

EM-12 monomer was obtained from our laboratory stock. The 2774-C10 human ovarian cancer cell line and LO2 human normal liver cells were provided by the

Sun Yat-sen University Cancer Center. Primary antibodies against cyclin E2, cyclin D1, CDK2, CDK6, caspase-8, caspase-3, and PARP were purchased from Cell Signaling Technology. The apoptosis detection kit was obtained from Becton, Dickinson and Company, the cell cycle detection kit from Jiangsu KeyGen Biotech Co., Ltd., and MTT reagent from Sigma-Aldrich.

1.2 Experimental Procedures

1.2.1 Cell Culture 2774-C10 and LO2 cells were cultured in DMEM supplemented with 10% fetal bovine serum at 37°C in a humidified incubator with 5% CO . Cells were passaged upon reaching 80% confluence using trypsin digestion.

1.2.2 MTT Assay for Cell Viability Logarithmically growing cells were seeded into 96-well plates at 3,500 cells per well in 100 L of cell suspension. After cell attachment, 100 L of drug dilution was added, with three replicate wells per group. Following 48 h of incubation, 10 L of MTT solution (5 g/L) was added to each well and incubated at 37°C for 4 h. The supernatant was removed, and 100 L of DMSO was added to dissolve the formazan crystals. Optical density was measured at 570 nm using a microplate reader. Cell viability was calculated as: (OD of experimental group / OD of control group) × 100%.

1.2.3 Colony Formation Assay Logarithmically growing 2774-C10 cells were trypsinized, resuspended in DMEM medium, and seeded into 6-well plates at 1,000 cells per well. After cell attachment, cells were treated with EM-12 or DMSO as a control. Following 7 days of culture, the supernatant was removed, cells were washed twice with PBS, fixed with 4% paraformaldehyde for 10 min, stained with 0.1% crystal violet for 30 min, rinsed, air-dried, and photographed.

1.2.4 GSEA Analysis Logarithmically growing 2774-C10 cells were seeded into 6-well plates and treated with EM-12 or DMSO control after attachment. After 24 h, the supernatant was removed, and cells were washed twice with PBS. Cells were lysed with 1 mL of TRIzol on ice for 5 min, after which the lysate was transferred to a new EP tube. Four hundred microliters of chloroform was added, mixed by vortexing, and allowed to stand at room temperature for 10 min. The mixture was centrifuged at 12,000 rpm for 15 min at 4°C. The aqueous phase was transferred to a new EP tube, mixed with an equal volume of isopropanol, and incubated at room temperature for 10 min. After centrifugation at 12,000 rpm for 15 min at 4°C, the supernatant was discarded, and the pellet was washed with 75% ethanol prepared with DEPC-treated water, air-dried, and dissolved in 30 L of DEPC water. RNA concentration was measured, and RNA-seq was performed using the BGI-500 platform. Sequencing data were subjected to GSEA analysis.

1.2.5 Flow Cytometry for Cell Cycle Analysis Logarithmically growing 2774-C10 cells were seeded into 6-well plates and treated with EM-12 or DMSO

control after attachment. After 24 h, the supernatant was removed, and adherent cells were collected by centrifugation at 1,500 rpm for 5 min. Cells were washed twice with PBS, fixed overnight in 70% ethanol, and then centrifuged again at 1,500 rpm for 5 min. After two PBS washes, cells were resuspended in 100 L of RNase A and incubated at 37°C for 30 min. Four hundred microliters of PI solution was added, and cells were incubated at 4°C in the dark for 30 min. Cells were filtered through a cell strainer, transferred to flow cytometry tubes, and analyzed.

1.2.6 Flow Cytometry for Apoptosis Detection Logarithmically growing 2774-C10 cells were seeded into 6-well plates and treated with EM-12 or DMSO control after attachment. After 48 h, both supernatant and adherent cells were collected and centrifuged at 1,500 rpm for 5 min. Cells were washed twice with PBS, resuspended in binding buffer, and incubated with 5 L of Annexin V-FITC and PI at room temperature in the dark for 15 min before flow cytometric analysis.

1.2.7 Western Blotting for Signaling Pathway Proteins Logarithmically growing 2774-C10 cells were seeded into 6-well plates and treated with EM-12 or DMSO control after attachment. After 24 h, the supernatant was removed, and cells were washed twice with PBS. Cells were lysed with 120 L of RIPA buffer on ice for 15 min, sonicated, and centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant was transferred to a new EP tube, and protein concentration was determined by BCA assay. Twenty-five micrograms of protein per sample were subjected to electrophoresis, transferred to membranes, and blocked. Membranes were incubated with primary antibodies overnight at 4°C, washed three times with TBST for 10 min each, incubated with secondary antibodies for 2 h at room temperature, washed again three times with TBST for 10 min each, and visualized using a Bio-Rad chemiluminescence system.

1.2.8 Statistical Analysis Statistical analysis was performed using SPSS 17.0 software. Data are presented as mean ± standard deviation. All experiments were performed independently three times. $P < 0.05$ was considered statistically significant.

Results

2.1 Cytotoxic Effects of EM-12 on Ovarian Cancer Cells

The cytotoxic effects of EM-12 on 2774-C10 ovarian cancer cells were evaluated using MTT assays, as shown in [Figure 2: see original paper]. The results demonstrated that EM-12 exerted significant cytotoxicity against 2774-C10 cells in a dose-dependent manner, with substantially lower cytotoxic effects on normal human liver LO2 cells.

2.2 Inhibition of Ovarian Cancer Cell Proliferation by EM-12

Following 7 days of EM-12 treatment, 2774-C10 cells showed a significant reduction in colony number and size compared to the control group. Control cells exhibited optimal growth with full, translucent morphology. At 0.5 mol/L EM-12, cell growth was impaired with cells beginning to round up. At 1 mol/L, only scattered cells remained visible, with colony formation nearly completely inhibited, as shown in Figure 3: see original paper.

2.3 EM-12 Induces G1/S Phase Arrest in 2774-C10 Cells

To identify signaling pathways affected by EM-12, we performed RNA-seq analysis on 2774-C10 cells treated with EM-12 for 24 h versus DMSO-treated controls. GSEA results revealed significant enrichment of gene sets related to G1/S phase arrest in EM-12-treated cells, as shown in Figure 4: see original paper. To confirm this finding, we analyzed cell cycle distribution by flow cytometry. PI staining demonstrated that increasing EM-12 concentrations led to a progressive increase in the G1-phase population and a corresponding decrease in S-phase cells, while G2-phase proportions remained largely unchanged. The G1/S ratio increased dose-dependently, with elevated pre-G1 populations, confirming that EM-12 induces G1/S phase arrest in 2774-C10 cells, as shown in Figure 4: see original paper.

2.4 EM-12 Induces Apoptosis in 2774-C10 Cells

Apoptosis was assessed using Annexin V-FITC/PI double staining. Treatment of 2774-C10 cells with varying concentrations of EM-12 for 48 h resulted in a dose-dependent increase in apoptosis rates, as shown in [Figure 5: see original paper].

2.5 Western Blot Analysis of Cell Cycle-Related Proteins

Based on GSEA and flow cytometry results indicating G1/S arrest, we examined the expression of key G1/S regulatory proteins including CDK2, CDK6, cyclin D1, and cyclin E2. Western blotting revealed that EM-12 treatment decreased the protein levels of CDK2, CDK6, cyclin D1, and cyclin E2 in a dose-dependent manner, as shown in Figure 6: see original paper, confirming G1/S phase arrest.

2.6 Western Blot Analysis of Apoptosis-Related Proteins

Flow cytometry analysis demonstrated EM-12-induced apoptosis, prompting further investigation of apoptosis pathway proteins. Apoptosis is a genetically controlled, orderly process of programmed cell death that maintains homeostasis. The fundamental mechanism involves reception of apoptotic signals, activation of caspase proteases through molecular interactions, and subsequent cleavage of PARP to execute cell death. Our results showed that EM-12 treatment activated caspase-8 and caspase-3 while inducing PARP cleavage in a concentration-

dependent manner, as shown in Figure 7: see original paper, demonstrating that EM-12 mediates apoptosis through the death receptor pathway.

Discussion

Elephantopus mollis H.B.K. is a medicinal plant belonging to the Asteraceae family, primarily distributed in Fujian, Guangdong, and other regions of China. It has been traditionally used for its heat-clearing, detoxifying, blood-cooling, and diuretic properties. Previous studies have reported the antitumor effects and molecular mechanisms of EM-3 and EM-23 isolated from this plant. However, the antitumor activities and mechanisms of other monomeric compounds from Elephantopus mollis remain unclear.

EM-12 is a monomeric compound extracted from the ethanol extract of Elephantopus mollis by our research group, and its antitumor effects and mechanisms have not been previously reported. Our findings demonstrate that EM-12 induces G1/S phase arrest and mediates apoptosis through the death receptor pathway in ovarian cancer 2774-C10 cells.

The G1/S checkpoint prevents cells from entering S phase upon DNA damage, allowing sufficient time for DNA repair and maintaining genomic stability. During G1/S transition, the CDK4/6-cyclin D complex promotes phosphorylation of pRB. Phosphorylated pRB undergoes conformational changes and dissociates from E2F, activating E2F and initiating expression of genes required for G1/S transition, including cyclin E. Cyclin E then forms a complex with CDK2 to further promote G1/S progression. Our study found that EM-12 significantly increased the proportion of 2774-C10 cells in G1 phase while decreasing cyclin D1, cyclin E2, CDK2, and CDK6 protein levels, indicating cell cycle arrest at the G1/S boundary.

Apoptosis is a genetically controlled process of programmed cell death that includes the extrinsic death receptor pathway, mitochondrial pathway, and endoplasmic reticulum stress-mediated pathway. The extrinsic death receptor pathway is initiated by TNF- binding to TNFR1, which activates downstream caspase-8 and releases its active fragments p18 and p10. Activated caspase-8 subsequently cleaves and activates caspase-3, which ultimately cleaves and inactivates PARP to execute apoptosis. Our study revealed that EM-12 activates caspase-8 and caspase-3 while inducing PARP cleavage, confirming that EM-12 mediates apoptosis through the death receptor pathway.

In conclusion, EM-12 inhibits the proliferation of ovarian cancer 2774-C10 cells, induces G1/S phase arrest, and triggers apoptosis via the death receptor pathway. These findings suggest promising therapeutic potential for EM-12 in ovarian cancer treatment and provide a theoretical foundation for its clinical application.

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Note: Figure translations are in progress. See original paper for figures.