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The natural terthiophene α-terthienylmethanol induces S phase cell cycle arrest of human ovarian cancer cells via the generation of ROS stress

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ABSTRACT

Ovarian cancer is the most lethal gynecological malignancy worldwide. Thiophenes such as terthiophene have been shown to have anti-tumor effects on several cancer cell lines, including ovarian cancer cells. However, the underlying mechanisms behind the anti-proliferative effect of thiophenes are poorly understood. In this study, we investigated the molecular mechanisms underlying the anti-proliferative effect of α-terthienylmethanol, a terthiophene isolated from *Eclipta prostrata* (False Daisy), on human ovarian cancer cells. We found that α-terthienylmethanol is a more potent inhibitor of cell growth than is cisplatin in human ovarian cancer cells. α-Terthienylmethanol induces cell cycle arrest in ovarian cancer cells, as shown by the accumulation of cells in S phase. In addition, α-terthienylmethanol induced a change in S phase-related proteins cyclin A, cyclin-dependent kinase 2, and cyclin D2. Knockdown of cyclin A using specific siRNAs significantly compromised α-terthienylmethanol-induced S phase arrest. We further demonstrated that α-terthienylmethanol induced an increase in intracellular ROS, and the antioxidant *N*-acetyl-L-cysteine significantly reversed the S phase arrest induced by α-terthienylmethanol. Moreover, α-terthienylmethanol significantly increased the levels of p-H2AX, a DNA damage marker. These results suggest that α-terthienylmethanol inhibits the growth of human ovarian cancer cells by S phase cell cycle arrest via induction of ROS stress and DNA damage.

1. Introduction

Ovarian cancer is the most lethal gynecological malignancy and the seventh leading cause of cancer death in women worldwide. More than 225,500 females are diagnosed with ovarian cancer every year worldwide [1]. This disease is deadly since most patients are diagnosed at late stages due to the absence of specific symptoms [2]. At present, the treatment for this cancer involves a combination of cytoreductive surgery and chemotherapy such as cisplatin. High-dose drug treatment may damage ovarian tissues as a result of the drug's side effects [3]. Overall survival rates are low due to drug resistance, which greatly limits future treatment. Therefore, the development of novel therapeutic agents is urgently needed for the treatment and survival of patients with ovarian cancer.

Cell replication results in a doubling of DNA and other cellular contents to duplicate a cell. This complex process is divided into four phases, including G1, S, G2, and M, which are defined by cell entry checkpoints. Passing these checkpoints determines whether the cell will proceed with division or stop. The major event of S phase is the replication of double-stranded DNA. External factors such as exposure to radiation, medications, and reactive oxygen species (ROS) result in DNA damage during S phase [4]. These DNA abnormalities halt the progression of the replication fork and inhibit the initiation of new replication origins until the damage is fixed. The G2 checkpoint maintains chromosomal integrity. During G2, which occurs between the S and M (mitosis) phases, DNA repair occurs before allowing the cell to enter the M phase. Cell division

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occurs during M phase once cells have passed all three restriction points. Cyclin-dependent kinases (CDKs), a family of serine/threonine kinases, control the mammalian cell cycle by binding to cyclins [5,6]. The cyclin A–CDK2 complex regulates the initiation of DNA replication during S phase [7]. Since DNA replication takes place during S phase, DNA damage is responsible for S phase arrest [8–10] as well as inhibition of cell proliferation. Therefore, a novel strategy to inhibit the growth of cancer cells is induction of DNA damage using DNA-damaging agents, leading to cell cycle arrest in S phase.

Eclipta prostrata is a perennial herb that belongs to the Asteraceae family and is widely distributed in tropical areas, especially China, Japan, Korea, and other Asian countries. Interestingly, *Eclipta prostrata* extract and its bioactive compounds have been shown to have anti-cancer activities. For example, *E. prostrata* extract showed cytotoxic effects in multiple cancer cell lines, including HepG2, C6, and A298 cells [11]. Eclalbasaponin I isolated from *E. prostrata* showed cytotoxic activity, with IC50 values of 111.17 μg/mL in liver cancer SMMC-7721 cells [12]. Saponin dayscyphin C from *E. prostrata* inhibited the growth of cervical HeLa cells, with an IC_{50} value of 50 μg/mL [13]. In a previous study, we isolated several thiophenes, which are sulfur-containing heterocyclic derivatives, from *E. prostrata* and showed that they are cytotoxic to human endometrial cancer cells. Thiophenes have been shown to possess various biological activities including anti-viral and anti-tumor activities [14–17]. However, the molecular mechanisms behind the anti-tumor and anti-proliferative effects of thiophenes are poorly understood in human cancer cells. In this study, we investigated the growth-inhibitory effects of α-terthienylmethanol on human ovarian cancer cells and the molecular mechanisms behind these effects.

2. Materials and methods

2.1. Materials

The whole plants of *Eclipta prostrata* (Asteraceae) were obtained from the Department of Pharmacy, Kyung Hee University Medical Center (Seoul, South Korea) in October 2011 and were identified by one of our authors, Prof. Dae Sik Jang. A voucher specimen (no. 2011-ECPR01) has been deposited in the Laboratory of Natural Product Medicine, College of Pharmacy, Kyung Hee University, Seoul, South Korea. The dried and milled plant material (400 g) was extracted with 2000 mL of 70% aqueous EtOH three times by maceration. The extracts were combined and concentrated in vacuo at 40 °C to give a 70% EtOH extract (45.2 g). A portion of the 70% EtOH extract (44 g) was suspended in distilled water (500 mL) and then successively extracted with *n*-hexane (3 × 500 mL), EtOAc (3 × 500 mL), and BuOH (3 × 500 mL) to give *n*-hexane- (2.86 g), EtOAc- (4.14 g), BuOH- (6.07 g), and water-soluble fractions (30.9 g), respectively. The *n*-hexane-soluble fraction was subjected to a column chromatography (CC) on silica gel (ϕ 3.8 × 36 cm^2, 70–230 mesh) eluted with *n*-hexane-EtOAc [(19:1, 9:1, 4:1, 7:3, 0:1 v/v, 1000 mL each eluent)] to afford 12 fractions (Fr.1–12). 5-Hydroxymethyl-(2, 2′:5′, 2″)-terthiophene (α-terthienylmethanol) (7.0 mg) was obtained from the fraction 9 [eluted with *n*-hexane–EtOAc (4:1 v/v); 230 mg] by a Sephadex LH-20 CC (ϕ 3.2 × 42 cm^2) with CH_2Cl_2–MeOH mixture (1:1 v/v) as mobile phase.

Roswell Park memorial Institute (RPMI) 1640, fetal bovine serum (FBS), penicillin and streptomycin were obtained from Life Technologies Inc. (Grand Island, NY, USA). 3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide (MTT) was purchased from Molecular Probes Inc. (Eugene, OR, USA). Propidium iodide (PI) was purchased from Sigma Chemical (St. Louis, MO, USA). Phenylmethylsulfonylfluoride (PMSF) and Annexin V-fluorescein isothiocyanate (FITC) were purchased from BD Biosciences (San Jose, CA, USA). Cyclin A, CDK2, cyclin D2, β-actin, and DCFH-DA (6-carboxyl-2, 7-dichlorodihydrofluorescein diacetate) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). H2AX, p-H2AX, and p-CDK2 were purchased from Cell Signaling (Beverly, MA, USA). Cyclin A small interfering RNA (siRNA) and the non-specific siRNA were purchased from BioNeer (Dae-gu, South Korea). Lipofectamine LTX™ and PLUS reagent were purchased from Invitrogen (Carlsbad, CA, USA).

2.2. Cell culture

Three human ovarian cancer cell lines A2780, SKOV3, OVCAR3, and ES2 cells were originally from American Type Culture Collection (Manassa, VA, USA). Ovarian cancer cells were cultured in RPMI 1640 supplemented with 5–10% fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin sulfate (100 μg/mL).

2.3. MTT assay

The cytotoxicity was analyzed by using MTT assay. Briefly, the cells (5×10^4) were seeded in each well containing 50 μL of RPMI medium in a 96-well plate. After 24 h, various concentrations of α-terthienylmethanol were added. After 48 h, 25 μL of MTT (5 mg/mL stock concentration) was added and the plates were incubated for an additional 4 h. The medium was discarded and the formazan blue salt, that generated in the cells, was dissolved in 50 μL DMSO. The optical density was measured at 540 nm using a microplate spectrophotometer (SpectraMax; Molecular Devices, Sunnyvale, CA, USA).

2.4. Flow cytometry assay

At the time of collection, the cells were harvested and washed twice with ice-cold PBS. The cells were fixed and permeabilized with 70% ice-cold ethanol at −20 °C for 4 h. The cells were washed once with PBS and resuspended in a staining solution containing propidium iodide (50 μg/mL) and RNase A (5 mg/mL). The cell suspensions were incubated for 30 min at room temperature in a dark place as propidium iodide is light sensitive. After 30 min the suspensions were analyzed by fluorescence-activated cell sorting (FACS) cater-plus flow cytometry (Becton Dickin-son Co., Germany) using 5000 cells per group. During apoptosis, exposure of phosphatidylserine on the exterior surface of the plasma membrane can be detected by the binding of fluoresceinated Annexin V (Annexin V-FITC). This assay is combined with analysis of the exclusion of the plasma membrane integrity probe PI. For annexin V and PI double staining, cells were suspended with 100 μL of binding buffer (10 mM HEPES/NaOH, 140 mM Nacl, 2.5 mM $CaCl_2$, PH 7.4) and stained with 5 μL of FITC-conjugated annexin V and 5 μL of PI (50 mg/mL). The mixture was incubated for 15 min at room temperature in a dark place and analyzed by FACS cater-plus flow cytometry.

2.5. Western blot analysis

Cells were washed with ice-cold PBS and extracted in protein lysis buffer (Intron, South Korea). Protein concentration was determined by a Bradford assay. Protein samples of cell lystate were mixed with equal volume of 5 × SDS sample buffer, denatured by heating for 5 min and then separated on 10% SDS-PAGE gels. After electrophoresis, proteins were transferred to polyvinylidene difluoride membranes. The membranes were blocked in 5% non-fat dry milk for 30 min, washed, and incubated with specific antibodies against cyclin A, cyclin D2, CDK2, p-CDK2, H2AX, p-H2AX, and β-