Original Article

Ent-11α-Hydroxy-15-oxo-kaur-16-en-19-oic-acid Inhibits Growth of Human Lung Cancer A549 Cells by Arresting Cell Cycle and Triggering Apoptosis

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ABSTRACT

Objective: To examine the apoptotic effect of ent-11α-hydroxy-15-oxo-kaur-16-en-19-oic-acid (5F), a compound isolated from *Pteris semipinnata* L (PsL), in human lung cancer A549 cells.

Methods: A549 cells were treated with 5F (0–80 μg/ml) for different time periods. Cytotoxicity was examined using a MTT method. Cell cycle was examined using propidium iodide staining. Apoptosis was examined using Hoechst 33258 staining, enzyme-linked immunosorbent assay (ELISA) and caspase-3 activity analysis. Expression of representative apoptosis-related proteins was evaluated by Western blot analysis. Reactive oxygen species (ROS) level was measured using standard protocols. Potential interaction of 5F with cisplatin was also examined.

Results: 5F inhibited the proliferation of A549 cells in a concentration- and time-dependent manner. 5F increased the accumulation of cells in sub-G1 phase and arrested the cells in the G2 phase. Exposure to 5F induced morphological changes and DNA fragmentation that are characteristic of apoptosis. The expression of p21 was increased. 5F exposure also increased Bax expression, release of cytochrome c and apoptosis inducing factor (AIF), and activation of caspase-3. 5F significantly sensitized the cells to cisplatin toxicity. Interestingly, treatment with 5F did not increase ROS, but reduced ROS production induced by cisplatin.

Conclusion: 5F could inhibit the proliferation of A549 cells by arresting the cells in G2 phase and by inducing mitochondrial-mediated apoptosis.

Key words: *Pteris semipinnata* L; Lung cancer; G2 cell cycle arrest; Apoptosis; Reactive oxygen species

INTRODUCTION

Lung cancer is one of the most common malignancies. Approximately 85% of lung cancer is non-small cell lung cancer (NSCLC). NSCLC is relatively insensitive to radio- and chemo-therapy. Also, in 75% of the cases, the cancer has already metastasized upon diagnosis[1, 2]. As a result, the 5-year overall survival rate is <15%[3]. More effective preventive and therapeutic approaches are urgently needed.

Ent-11-hydroxy-15-oxo-kaur-16-en-19-oic acid (5F) is an active compound in *Pteris semipinnata* L (PsL)[4-6]. Earlier studies from our laboratory demonstrated 5F could inhibit the *in vitro* growth of a number of tumors (e.g., colorectal cancer, gastric cancer, anaplastic thyroid carcinoma, liver cancer and laryngeal cancer) via a mitochondrial- mediated apoptotic mechanism[7-11]. We have also compared 5F with 7-hydroxystaurosporine (UCN01), which is currently being evaluated in clinical trials as an antitumor drug in the United State and Japan[12,13]. UCN01 is an inhibitor of protein kinase C, but has higher specificity than its parent compound staurosporine. Our previous study demonstrated equal cytotoxicity of 30 nmol/L 5F to 100 nmol/L UCN01 in colorectal cancer cells. Both UCN01 and 5F increased p38 mitogen-activated protein kinase (MAPK) and nitric oxide (NO) levels. Overexpression of either Bcl-2 or Bcl-xL prevented UCN01-induced cell death. Over-expression of Bcl-2, but not Bcl-xL protected the cells

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from 5F[7]. These results indicated overlapping yet different antitumor mechanisms for 5F and UCN01.

A recent study in mice from this laboratory showed that 5F is also effective against lung cancer induced by 4-methylnitrosamino-1-3-pyridyl- butanone (NNK) with minimal side effects[14]. The present study examined the potential antitumor action of 5F in a human NSCLC cell line, A549. Possible interaction between 5F and cisplatin was also examined.

MATERIALS AND METHODS

Antibodies and Reagents

Mouse-anti-human monoclonal primary antibodies for β-actin, Bax, cytochrome c, apoptosis inducing factor (AIF), p21 and horseradish peroxidase-conjugated goat-anti- mouse IgG antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). RPMI 1640, fetal bovine serum (FBS), 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), propidium iodide (PI) and Hoechst 33258 were purchased from Sigma-Aldrich (St. Louis, MO, USA). 5F was isolated from PsL as described previously[15]. All other reagents were purchased from Sigma-Aldrich unless otherwise mentioned.

Cell Culture

A549 cells were obtained from the American Type Culture Collection and cultured in RPMI 1640 medium containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were cultured at 37°C in a humidified 5% CO_2 incubator. 5F was dissolved in propylene glycol (PG) and diluted with the culture medium immediately before use (final PG concentration ≤1.2%). In all experiments, the cells in RPMI 1640 medium plus PG only were used as the control.

Cell Viability Assay

Cells were seeded in a 96-well plate at a density of 5×10^3 cells/well. The total volume was adjusted to 200 μl with growth medium. At 24 h after the seeding, the cells were exposed to 5F (0–80 μg/ml), or a combination of 5F (10 μg/ml) and cisplatin (10 μg/ml). Cell viability was examined after 24, 48 or 72 h using a standard MTT method. Drug effect was expressed as percentage relative to the controls. Morphology of the cells was examined after 24 h exposure to 5F under an inverted phase contrast microscope.

Hoechst 33258/PI Staining Assay

Cells (1×10^5) were seeded onto cover slides and treated with 5F (0–80 μg/ml) for 24 h, then washed with ice-cold PBS and stained with Hoechst 33258 (10 μg/ml) at 37°C for 15 min. After removing free Hoechst 33258 with PBS, cells were stained with PI (20 μg/ml) at 4°C for 15 min, and washed again with PBS prior to observation under a fluorescence microscope (DMLB/MPS-30/Q500IW, LEICA, Germany).

Cell Cycle Distribution

A549 cells were seeded at a density of 1×10^5 cells/well in a six-well plate. At 24 h after the seeding, the cells were treated with 5F (0–80 μg/ml) for 24 h at 37°C. Cells were fixed overnight with 70% ethanol at –20°C and stained with PI solution (100 μg/ml). Cell cycle distribution analysis was performed using a flow cytometer (EPICS XL, COULTER, USA).

Apoptosis Enzyme-linked Immunosorbent Assay (ELISA)

Cytoplasmic histone-associated-DNA-fragments (mono- and oligonucleosomes) were determined by Apoptosis Detection ELISAPLUS (Roche, Mannheim, Germany). Briefly, 1×10^4 cells/well were seeded in a 96-well plate and treated with various concentrations (0–80 μg/ml) of 5F for 24 h. After 24 h incubation, cells were lysed and centrifuged at 1,500 r/min. The cytoplasmic fraction (the supernatant) containing fragmented DNA was transferred to a streptavidin-coated microplate. A mixture of anti-histone-biotin and anti-DNA-peroxidase was added and incubated for 2 h at room temperature. After removal of unbound antibodies by washing, the amount of fragmented DNA of nucleosomes bound to anti-histone-biotin was evaluated by anti-DNA- peroxidase using peroxidase substrate at 405 nm.

Measurement of Caspase-3 Activity

Caspase-3 activity was measured using a spectrometric method as specified in the assay kit instruction (Caspase-3 Colorimetric Assay Kit, KeyGen Biotech, Nanjing, China). Cells were treated with 5F (0–80 μg/ml) for 24 h prior to harvest. Fifty microliter lysis buffer [containing 1% dithiothreitol (DTT)] was added to $(3–5)\times10^6$ cells. The cells were incubated for 60 min on ice, and vortexed 3–4 times during the incubation. The lysate was centrifuged at 10,400 r/min for 1 min. Protein concentration in the supernatant was measured. Fifty microliter lysate supernantant (containing 100 μg protein) was mixed with 50 μl 2× reaction buffer (containing 1% DTT) and 50 μl caspase-3 substrate, and incubated for 4 h at 37°C in darkness. Absorbance was measured at 405 nm. Caspase-3 activity was expressed as: $OD_{5F}/OD_{control}$.

Measurement of Reactive Oxygen Species (ROS)

Intracellular ROS was measured using a spectrometric method as specified by manufacturer's instruction (ROS Assay Kit, GENMED Scientific, Shanghai, China). Cells (1×10^4 cells/well; 96-well plate) were treated with the test agent for 3 h prior to addition of Reagent A (20 μl/well) and subsequent incubation for 60 min at 37°C. The supernatant was aspirated prior