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Cytotoxic activities and effects of atractylodin and β-eudesmol on the cell cycle arrest and apoptosis on cholangiocarcinoma cell line

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ABSTRACT

Cholangiocarcinoma (CCA) is the cancer of bile duct with high mortality rate particularly in Thailand. The clinical efficacy of the standard chemotherapeutics remains unsatisfactory, and therefore, discovery and development of the new alternative drugs with high efficacy and tolerability is needed. The aim of the study was to investigate cytotoxic activity as well as the underlying mechanisms through which atractylodin and β-eudesmol exert their activities on CCA cell growth inhibition, cell cycle arrest, and cell apoptosis. Effects of the compounds on cell cytotoxicity, cell cycle arrest, and cell apoptosis were analyzed using MTT assay, BD Cycletest™ Plus DNA kit, and FITC Annexin V Apoptosis Detection Kit I, respectively. The cytotoxic activities of both compounds were concentration- and time-dependent. The IC_{50} [mean (SD)] of atractylodin and β-eudesmol were 41.66 (2.51) and 39.33 (1.15) μg/ml respectively. Both promoted cell cycle arrest at G1 phase, and induced cell apoptosis through activation of caspase-3/7. The highest activity was observed at 48 h of exposure. Results suggest that these mechanisms are at least in part, explain the cell cytotoxic and anti-CCA activity of atractylodin and β-eudesmol shown *in vitro* and *in vivo* models.

1. Introduction

Cholangiocarcinoma (CCA) is the cancer of bile duct arising from epithelial cells and develops along the biliary tree. CCA is classified in several types based on anatomical location, microscopic pattern of growth, microscopic feature, and origin cell.[1] In the United States, CCA accounts for 3% of all gastrointestinal cancers with approximately 5000 new cases annually.[2] The highest incidence and mortality rates are reported from Thailand, particularly in the northeastern region of the country. Current standard treatments of CCA with conventional drugs include 5-fluorouracil (5-FU), gemcitabine, and cisplatin, either as single drug or combination therapies.[3,4] Nevertheless, their clinical efficacy remains unsatisfactory, and therefore, discovery and development of the new alternative drugs with high efficacy and tolerability is needed.

The rhizome of *Atractylodes lancea* (*A. lancea*) is used ethnobotanically in China, Japan and Thailand for treatment several diseases or conditions such as rheumatic, digestive disorders, night blindness, influenza, fever, and common cold.[5] Moreover, various studies also demonstrated potential property of the rhizome extract of this plant for treatment of cancers.[6–8] The anticancer potential of the rhizome extract of *A. lancea* against CCA was also reported both *in vitro* and *in vivo* models.[9–13] In the present study, we further investigated the cytotoxic activity as well as the underlying mechanisms through which the two major isolated compounds, *i.e.*, atractylodin (Fig. 1A, 14% of total content) and β-eudesmol (Fig. 1B, 6% of total content) exert their activities on CCA cell growth inhibition, cell cycle arrest, and cell apoptosis.

2. Material and methods

2.1. Cell lines and culture

The CCA cell lines (CL-6 and HUCC-T1), and normal (OUMS) human cell line were used in the study. The CL-6 cell originally isolated from a patient with CCA, was kindly provided by Associate Professor Dr. Adisak Wongkajornsilp, Faculty of Medicine, Siriraj

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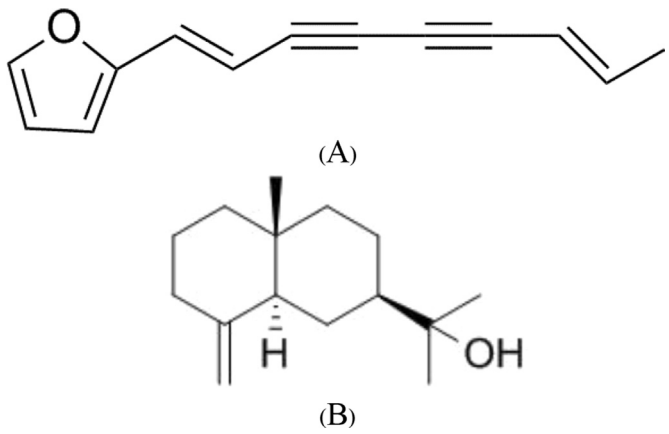

Fig. 1. The chemical structures of atractylodin (A) and β-eudesmol (B).

Hospital, Mahidol University, Thailand. HUCC-T1 and OUMS were purchased from Japanese Collection of Research Bioresources (JCRB) cell bank, Japan. All cell lines were cultured with RPMI 1640 medium (Gibco Co. Ltd., NY, USA) and Dulbecco's Modified Eagle Medium (DMEM: Gibco Co. Ltd., NY, USA), respectively. The culture medium was supplemented with 10% (v/v) heated fetal bovine serum (FBS) and 100 IU/ml of antibiotic-antimycotic solution (Gibco Co. Ltd., NY, USA). All cells were maintained at 37 °C in 5% CO_2 atmosphere and 95% humidity (HERACELL 150i, Thermo scientific, MA, USA).

2.2. Cytotoxic assay

The CCA, HUCC-T1, and OUMS cell lines were treated with varying concentrations (250, 125, 62.5, 31.25, 15.6, 7.8, 3.9, and 1.95 μg/ml) of atractylodin, β-eudesmol, and 5-FU (WAKO, Osaka, Japan) in a 96-well microtiter plate (Corning, NY, USA) for 48 h 5-FU (WAKO, Osaka, Japan) was used as a positive control (concentration range 250−1.95 μg/ml). Effects of all compounds on the survival of all cell lines were determined using MTT assay.[14] Briefly, the cells (CL-6, HUCC-T1, or OUMS) were seeded onto each well of the 96-well microtiter plate (10,000 cells/well) and incubated for 24 h (37 °C, 5% CO_2 atmosphere, and 95% humidity) before exposing to atractylodin, β-eudesmol, or 5-FU. Following 48 h incubation, the MTT reagent (20 μl of 5 mg/ml solution: Sigma Co. Ltd., MO, USA) was added into each well and the plate was further incubated for 4 h. The culture medium of each well was discarded and DMSO (100 μl) was added, and the plate was incubated at 25 °C in the dark room for 15 min. The absorbance was measured at 570 nM (Varioscan Flash, Thermo, Finland).

Cell viability and corresponding IC_{50} (concentration of each compound that produces 50% inhibitory effect on cell growth relative to control) were determined using CalcuSyn™ v2.11 software (Biosoft, Cambridge, UK). The selectivity index (SI) was determined as the ratio of IC_{50} of atractylodin, β-eudesmol, or 5-FU in the OUMS and that in the CCA cell.

2.3. Effect of time on cytotoxic activity of atractylodin and β-eudesmol

To determine the effect of time on the cytotoxic activity of atractylodin and β-eudesmol in CL-6, HUCC-T1, and OUMS cells, the cells were seeded onto a 96-well plate (10,000 cells/well) and incubated for 24 h. Following exposure to atractylodin or β-eudesmol at the IC_{50} concentration of each compound for 12, 24, and 48 h, cell viability was determined using MTT assay as described above.

2.4. Effects of atractylodin and β-eudesmol on cell cycle arrest

The CL-6, HUCC-T1, and OUMS cells were exposed to atractylodin and β-eudesmol at the IC_{25} (concentration of each compound that produces 25% inhibitory effect relative to control) of each compound (20 and 23 μg/ml for atractylodin and β-eudesmol, respectively) for 12, 24, and 48 h and DNA contents were identified by BD Cycletest™ Plus DNA kit (BD biosciences, CA, USA). Briefly, the cells were harvested after the exposure to each compound, washed three times with buffer solution, and cell number was adjusted to 1.0×10^6 cells/ml. Following sequential incubation (10 min each) in the dark room with Solution A (trypsin buffer), Solution B (trysin inhibitor and RNase buffer), and Solution C (PI stain solution), the deoxyribonucleic acid (DNA) content of CL-6 was analyzed by flow cytometry (BD FACSVerse™ flow cytometer, BD, USA). Each study was performed in three independent experiments, triplicate each.

2.5. Effects of atractylodin and β-eudesmol on cell apoptosis and caspase 3/7 activation

The CL-6, HUCC-T1, and OUMS cells were exposed to atractylodin and β-eudesmol at the IC_{25} and IC_{50} concentrations of each compound (IC_{25}: 20 and 23 μg/ml for atractylodin and β-eudesmol, respectively; IC_{50}: 40 and 40 μg/ml for atractylodin and β-eudesmol, respectively) for 12, 24, and 48 h, and cell apoptosis and caspase-3/7 activation was investigated using FITC Annexin V Apoptosi Detection Kit I and CellEvent™ Caspase-3/7 Green Flow Cytometry Assay Kit. Briefly, the cells were harvested after exposure to each compound, washed three times with PBS solution, and cell number was adjusted to 1.0×10^5 cells/ml. The cells were mixed with CellEvent™ Caspase-3/7 Green Detection Reagent and incubated at 37 °C for 30 min in the dark room. Finally, the cells were mixed with SYTOX™ AADvanced™ in DMSO and analyzed by flow cytometry (BD FACSVerse™ flow cytometer, BD, USA). Each study was performed in three independent experiments, triplicate each.

3. Results

3.1. Cytotoxic activity

The IC_{50} [mean (SD)] values of atractylodin in CL-6, HUCC-T1, and OUMS cell lines were 41.66 (2.51), 38.78 (1.07) and 64.33 (2.08) μg/ml, respectively. The corresponding values of the IC_{50} for β-eudesmol were 39.33 (1.15), 35.22 (0.98) and 53.15 (3.90) μg/ml, respectively. The SI values of both compounds for the CL-6 vs. HUCC-T1 cells were 1.54 vs. 1.66 and 1.35 vs. 1.50, respectively.

3.2. Effect of time on cytotoxic activity of atractylodin and β-eudesmol

Both atractylodin and β-eudesmol exhibited time-dependent cytotoxic activity in both CL-6 and HUCC-T1 cells over the exposure period 12−48 h. The IC_{50} [mean (SD)] values of atractylodin following 12, 24, and 48 h exposure of the CL-6 vs. HUCC-T1 cells were 80.33 (4.50) vs. 76.41 (2.22), 66.33 (3.05) vs. 60.20 (2.79), and 41.66 (2.51) vs. 37.00 (1.22) μg/ml, respectively. The corresponding IC_{50} [mean (SD)] values of β-eudesmol were 61.33 (2.51) vs. 58.29 (2.01), 50.00 (2.00) vs. 45.11 (3.02) and 39.33 (1.15), 35.11 (1.09) μg/ml, respectively (Fig. 2A and B).