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Guanylate cyclase activator YC-1 potentiates apoptotic effect of licochalcone A on human epithelial ovarian carcinoma cells via activation of death receptor and mitochondrial pathways

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ABSTRACT

Natural phenol licorice compounds have been shown to induce apoptosis in cancer cells. 3-(5′-Hydroxymethyl-2′-furyl)-1-benzyl indazole (YC-1) may enhance the sensitivity of cancer cells to anticancer drugs. However, the combined effect of licochalcone A and YC-1 on cell death in ovarian cancer cells has not been studied. We assessed the combined effect of licochalcone A and YC-1 on apoptosis in human epithelial ovarian carcinoma cell lines in relation to the cell death process. In the OVCAR-3 and SK-OV-3 cell lines, licochalocone A induced a decrease in Bid, Bcl-2, Bcl-xL and survivin protein levels; an increase in Bax levels; loss of the mitochondrial transmembrane potential; cytochrome *c* release; activation of caspases (-8, -9 and -3); cleavage of PARP-1; and an increase in the tumor suppressor p53 levels. YC-1 enhanced licochalcone A-induced apoptosis-related protein activation, nuclear damage and cell death. These results suggest that YC-1 may potentiate the apoptotic effect of licochalcone A on ovarian carcinoma cell lines by increasing the activation of the caspase-8- and Bid-dependent pathway and the mitochondria-mediated apoptotic pathway, leading to caspase activation. The combination of licochalcone A and YC-1 may confer a benefit in the treatment of human epithelial ovarian adenocarcinoma.

1. Introduction

YC-1 (3-(5′-hydroxymethyl-2′-furyl)-1-benzyl indazole) is a small molecule that functions as a guanylate cyclase activator through direct binding with the enzyme. YC-1 is used as an anticancer drug, likely due to its inhibitory effect against hypoxia inducible factor, which is involved in tumor growth, vascularization and metastasis (Pan et al., 2005; Wu et al., 1995; Yeo et al., 2003). YC-1 exhibits anti-proliferative effects against various cancer cell lines by inducing cell cycle arrest, apoptosis, anti-angiogenesis and inhibition of matrix metalloproteinases (Chen et al., 2008; Pan et al., 2005; Yeo et al., 2003; Zhao et al., 2007). It enhances the chemo-sensitivity of cancer cells to cisplatin or camptothecin via changes in apoptosis-related protein levels, leading to the activation of caspases (Lau et al., 2007; Lee et al., 2009). Further, YC-1 increases hypoxia-induced cell cycle arrest and death in Hep3B hepatoma cells, Caki-1 renal carcinoma cells and pancreatic cancer cells (Yeo et al., 2006; Zhao et al., 2007). Alternatively, YC-1 prevents glutamate-induced apoptosis in differentiated PC12 cells (Yang et al., 2011) and inhibits oxygen/glucose deprivation-induced axonal damage (Garthwaite et al., 2002).

Licorice compounds such as licochalcone A, glycyrrhizin and 18β-glycyrrhetinic acid (a hydrolyzed metabolite of glycyrrhizin) have anti-inflammatory, anticancer and antiviral effects (Cui et al., 2008; Fu et al., 2004; Hoever et al., 2005; Matsui et al., 2004; Shibata, 2000). Licochalcone A inhibits TNF-alpha-induced nuclear factor-κB activation, leading to decrease in the production of inflammatory cytokines (Funakoshi-Tago et al., 2009) and reduces paw edema induced by carrageenan (Cui et al., 2008). Licochalcone A induces growth inhibition and apoptosis in prostate cancer cells (Fu et al., 2004) and gastric cancer cells (Xiao et al., 2011). It also inhibits angiogenesis and metastasis in mouse colon cancer models (Kim et al., 2010; Lee et al., 2008b). However, the licorice compounds differentially alter the effects of anticancer drugs on cancer cells depending upon the type of anticancer drug (Lee et al., 2008a). In addition, it is unclear if the mechanism of licorice compound toxicity is mediated by the activation of the cell surface death receptor Fas (Ishiwata et al., 1999; Yoshikawa et al., 1999). Furthermore, licorice compounds can have opposing effects; glycyrrhizin exhibits a pro-apoptotic effect

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against bile acid-induced cell death in rat hepatocytes, whereas 18β-glycyrrhetinic acid potently inhibits it (Gumpricht et al., 2005).

The conventional anticancer drugs platinum and paclitaxel are used for the treatment of epithelial ovarian cancer. However these drugs cause serious toxicity and problems, such as myelosuppression and resistance, and thus new drug therapies are needed (Bookman, 2003). The licorice compounds have been shown to induce apoptosis in cancer cells, but the apoptotic effect of licochalcone A on ovarian cancer cells remains uncertain. YC-1 may enhance the sensitivity of cancer cells to anticancer drugs, but the combined effect of licochalcone A and YC-1 on cell death in ovarian cancer cells has not been studied. Therefore, we assessed the combined effect of licochalcone A and YC-1 on apoptosis in human epithelial ovarian carcinoma cell lines in relation to the cell death process.

2. Materials and methods

2.1. Materials

The TiterTACS™ colorimetric apoptosis detection kit was purchased from Trevigen, Inc. (Gaithersburg, MD). The assay kits for human cytochrome *c* (Quantikine® M) and caspases (-8, -9 and -3) were purchased from R&D systems (Minneapolis, MN). The antibodies (anti-Bid (5C9), anti-Bax (6A7), anti-Bcl-2 (10C4), anti-Bcl-xL (H-5), anti-survivin (D-8), anti-cytochrome *c* (A-8), PARP-1 (B-10), anti-p53 (DO-1) and anti-β-actin) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The licochalcone A, 3-(5′-hydroxymethyl-2′-furyl)-1-benzyl indazole (YC-1), horseradish peroxidase-conjugated anti-mouse IgG, z-Asp-(OMe)-Gln-Met-Asp(OMe) fluoromethyl ketone (z-DQMD.fmk) and z-Ile-Glu-(O-ME)-Thr-Asp(O-Me) fluoromethyl ketone (z-IETD.fmk) were all purchased from EMD-Calbiochem (La Jolla, CA). The Super-Signal® West Pico chemiluminescence substrate for the cytochrome *c* detection in western blotting was purchased from PIERCE Biotechnology Inc. (Rockford, IL). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), z-Leu-Glu-(O-ME)-His-Asp(O-Me) fluoromethyl ketone (z-LEHD.fmk), 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6(3)$) and other chemicals were purchased from Sigma-Aldrich Inc. (St. Louis, MO).

2.2. Cell culture

The NIH-OVCAR-3 and SK-OV-3 cell lines (origin: human ovary; cellular morphology: epithelial; histopathology: adenocarcinoma) were obtained from the Korean cell line bank (Seoul, South Korea) and cultured in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml of penicillin and 100 μg/ml of streptomycin, according to the directions provided by the cell bank. Cells were washed with RPMI medium containing 1% FBS 24 h before the experiments and were seeded in 96- or 24-well plates.

2.3. Cell viability assay with MTT reduction

Cell viability was measured using the MTT assay, which is based on the conversion of MTT to formazan crystals by mitochondrial dehydrogenases (Mosmann, 1983). Cells (3×10^4) were treated with licochalcone A for 24 h at 37 °C. The medium (200 μl) was then incubated with 10 μl of 10 mg/ml MTT solution for 2 h at 37 °C. After centrifugation at $412\times g$ for 10 min, the culture medium was removed, and 100 μl of dimethyl sulfoxide was added to each well to dissolve the formazan. The absorbance was measured at 570 nm using a microplate reader (Magellan, TECAN, Salzburg, Austria). Cell viability is expressed as a percentage of the absorbance value determined for the control cultures.

2.4. Cell viability assay with neutral red uptake

Cell viability was determined using the neutral red uptake assay, which is based on the observation that neutral red is accumulated in the lysosomes of live cells (Andrisano et al., 2001). OVCAR-3 cells (2×10^4) were treated with licochalcone A for 24 h at 37 °C. The cell suspension (200 μl) was then incubated with 10 μl of 1 mg/ml neutral red solution for 3 h at 37 °C. After centrifugation at $412\times g$ for 10 min, the culture medium was removed, and the dye was extracted with 100 μl of a 1% acetic acid and 50% ethanol solution for 20 min. The absorbance was measured at 540 nm using a microplate reader.

2.5. Observation of changes in nuclear morphology

OVCAR-3 cells (1×10^6 cells/ml) were treated with licochalcone A for 24 h at 37 °C, and the changes in nuclear morphology were assessed using Hoechst dye 33258 (Oberhammer et al., 1992). Cells were incubated with 1 μg/ml Hoechst 33258 for 3 min at room temperature and the nuclei were visualized using an Olympus microscope with a WU excitation filter (Tokyo, Japan).

2.6. Measurement of oligonucleosomal DNA fragmentation

DNA fragmentation due to the activation of the endonucleases was assessed by gel electrophoresis. OVCAR-3 cells (4×10^6 cells/ml) were treated with licochalcone A for 24 h at 37 °C and then washed with PBS. The DNA was isolated with the DNA purification kit, according to the manufacturer instructions (Wizard® Genomic, Promega Co., WI). The DNA pellets were loaded on a 1.5% agarose gel in Tris-acetate buffer (pH 8.0) and 1 mM EDTA and were separated at 100 V for 2 h. The DNA fragments were stained with ethidium bromide and then visualized using a UV transilluminator.

2.7. Quantitative analysis of DNA fragmentation

DNA fragmentation during apoptosis was assessed using a solid-phase enzyme-linked immunosorbent assay (ELISA). OVCAR-3 cells (3×10^4) were treated with licochalcone A for 24 h at 37 °C, washed with phosphate-buffered saline (PBS) and fixed with formaldehyde solution. Deoxynucleotides (dNTPs) were incorporated at the 3′-ends of the DNA fragments by terminal deoxynucleotidyl transferase (TdT) and the nucleotides were detected using streptavidin-horseradish peroxidase and TACS-Sapphire according to the TiterTACS protocol. Data are expressed as the absorbance at 450 nm.

2.8. Western blot analysis

To assess the effect of YC-1 on the licochalcone A-induced apoptosis-related protein activation as an early phenomenon, we investigated the effects after a 4 h-incubation. Cell lines (5×10^6 cells/ml) were harvested by centrifugation at $412\times g$ for 10 min, washed twice with PBS and suspended in lysis buffer A (250 mM sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 0.5 mM dithiothreitol, 0.1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 20 mM HEPES-KOH, pH 7.5). The lysates were further homogenized by successive passages through a 26-gauge hypodermic needle. The homogenates were centrifuged at $100{,}000\times g$ for 5–30 min depending on the protein that was being detected, and the supernatant was used for western blotting and ELISA. The protein concentration was determined using the Bradford method, according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA).

Cell mitochondrial fraction was isolated using the digitonin lysis method (Dai et al., 2009; Wu et al., 2000). Briefly, cells (5×10^6) were incubated with ice-cold digitonin lysis buffer (250 mM sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 0.5 mM dithiothreitol, 0.1 mM PMSF, 190 μg/ml digitonin and 20 mM HEPES-KOH,