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Ganoderiol F, a ganoderma triterpene, induces senescence in hepatoma HepG2 cells

Ue-Min Chang [a], Chyi-Hann Li [c], Liang-In Lin [a,b], Cheng-Po Huang [a], Lou-Sing Kan [c,*], Shwu-Bin Lin [a,b,*]

[a] *Department of Clinical Laboratory Sciences and Medical Biotechnology, College of Medicine, Taiwan ROC*
[b] *Department of Laboratory Medicine, National Taiwan University Hospital, National Taiwan University, Taiwan ROC*
[c] *Institute of Chemistry, Academia Sinica, Taipei, Taiwan ROC*

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Abstract

Ganoderiol F (GolF), a tetracyclic triterpene, was isolated from *Ganoderma amboinense* and found to induce senescence of cancer cell lines. GolF induced growth arrest of cancer cell lines HepG2, Huh7 and K562, but exerted much less effect in hepatoma Hep3B cells and normal lung fibroblast MRC5 cells, and no effect on peripheral blood mononuclear cells. GolF treatment of the cancer cells, with the exception of Hep3B, resulted in prompt inhibition of DNA synthesis and arrest of cell progression cycle in G1 phase. Short-term exposure of HepG2 cells to GolF temporarily arrested progression of the cell cycle; cell growth was recovered if the drug was withdrawn from the medium after a 24-h exposure. After 18 days of continuous treatment of HepG2 cells with 30 μM GolF, over 50% of cells were found to be enlarged and flattened, and were β-galactosidase positive phenotypes of senescent cells. GolF was found to inhibit activity of topoisomerases in vitro, which may contribute to the inhibition of cellular DNA synthesis. Activation of the mitogen-activated protein kinase EKR and up-regulation of cyclin-dependent kinase inhibitor p16 were found in early stages of GolF treatment and were presumed to cause cell-cycle arrest and trigger premature senescence of HepG2 cells. The growth-arrest and senescence induction capability on cancer cells suggest anticancer potential of GolF.

Keywords: Ganoderiol F; Ganoderma triterpene; Cell senescence; G1 cell-cycle arrest; p16; pERK

Introduction

In long-lived multi-cellular organisms, senescence of normal cells is a mechanism contributing to oppose neoplastic transformation (Lloyd, 2002). Senescent cells are arrested in the G1 phase of the cell cycle, typically appear enlarged and flattened in shape, with increased cytoplasmic granularity and they express senescence associated β-galactosidase (SA-β-gal) (Dimri et al., 1995). It has been shown that cell senescence as the result of cells responding to cellular stress, e.g. shortening of telomeres, DNA damage or mitogenic signals, is controlled by tumor suppressor proteins such as retinoblastoma protein (pRb) or p53, and constitutes a potent anticancer mechanism (Itahana et al., 2004; Shay and Roninson, 2004). Cancer cells might be induced to undergo senescence through expression of tumor suppressor genes or inhibition of oncogenes (Campisi, 2005; Mathon and Lloyd, 2001; Goodwin et al., 2000). It has been shown that some anticancer agents may induce long-term growth arrest and consequently senescence of cancer cells. Activation of the senescence program in cancer cells seems to be an alternative therapeutic strategy other than induction of apoptosis (Chang et al., 1999; Roninson et al., 2001).

Fruit bodies of ganoderma fungi have been used in traditional Chinese medicine for a long time and are believed to have anti-cancer and immunomodulatory activities. The medicinal mushroom is also taken in tonics and remedies for ailments such as cough, asthma, insomnia, indigestion, bronchitis, hepatitis and hypertension (Yan et al., 1999). The

* Corresponding authors. Lou-sing Kan is to contacted at Institute of Chemistry, Academia Sinica, Taipei, Taiwan R.O.C. Fax: +886 2 27884184. Shwu-Bin Lin, 1 Chang-Te Street, 10016 Taipei, Taiwan, ROC. Fax: +886 2 23711574.
E-mail addresses: lskan@chem.sinica.edu.tw (L.-S. Kan), sblin@ntumc.org (S.-B. Lin).

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major bioactive ingredients of the fungus include polysaccharides, glycoproteins, and triterpenes. Up to now, over a hundred ganoderma triterpenes have been identified. Some of them were characterized to have pharmacological functions including hepato-protection, inhibition of histamine release, inhibition of cholesterol synthesis, stimulation or inhibition of platelet aggregation, anti-HIV, antitumor, anti-angiogenic, anti-metastasis, and anti-angiogenesis (Kimura et al., 2002; Shiao, 2003). We have previously found that triterpene enriched fraction from *Ganoderma lucidum* inhibits the growth of cancer cells by modulating activities of protein kinase C and mitogen-activated protein (MAP) kinases (Lin et al., 2003). Several triterpenes were purified from fruit bodies of *Ganoderma amboinense* and one of them was found to induce apoptosis of cancer cell lines, presumably through inhibiting the activities of topoisomerases (Li et al., 2005). In this report another triterpene isolate, ganoderiol F (GolF), was found to arrest cell growth and consequently induce senescence in hepatoma HepG2 cells. GolF-induced cell senescence might be mediated by the activation of ERK MAP kinase and the induction of cyclin-dependent kinase (CDK) inhibitor p16, a tumor suppressor protein.

Materials and methods

Materials

Human topoisomerase (topo) I, topo IIα and Ficoll-Hypaque were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Kinetoplast DNA (kDNA) and pRYG plasmid DNA were purchased from TopoGEN Inc. (Columbus, OH, USA). Antibodies for p38, JNK and ERK were purchased from Santa Cruz (Santa Cruz, CA, USA) and those for the phosphorylated forms of p38, JNK and ERK (p-p38, p-JNK and p-ERK) were from Cell Signaling (Beverly, MA, USA). Antibody for actin was obtained from Chemicon (Temecula, CA, USA). The DNA primers were obtained from Mission Biotech (Taipei, Taiwan). Fruit bodies of *G. amboinense* were purchased from a Chinese medicine store in Taipei, Taiwan. GolF was purified according to the protocol described previously from methanol extract of the fruit bodies using two runs of silica gel chromatography followed by a cycle of reverse phase HPLC (Li et al., 2005). The isolation and characterization of GolF will be published elsewhere.

Topoisomerase assay

The relaxation activities of topo I and topo IIα were determined by analyzing the conversion of supercoiled plasmid DNA into its relaxed forms. The topo I reaction was performed in 20 μl reaction buffer containing 35 mM Tris–HCl (pH 8.0), 72 mM KCl, 5 mM $MgCl_2$, 5 mM DTT, 5 mM spermidine, 0.01% bovine serum albumin (BSA), 2 units of topo I and 400 ng supercoiled pRYG plasmid DNA. GolF was added to the reactions to desired concentration. After incubation for 30 min at 37 °C, the reaction was terminated by heating at 75 °C for 5 min and addition of 4 μl loading dye containing 5 mM EDTA, 30% glycerol, 5% SDS, and 0.2% each of bromophenol blue and xylene cyanol. Relaxation activity of topo IIα was analyzed in the same manner, except that the reaction mixtures contained 10 mM Tris–HCl (pH 7.9), 50 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 15 μg/ml BSA, 1 mM ATP and 3 units topo IIα. Decatenation activity of topo IIα was assessed under the same conditions except that the supercoiled plasmid DNA was replaced with 400 ng kDNA. After reaction, the DNA was separated on 1.2% agarose gels in 0.5 × TPE at 50 mV for 2.5 h. The gels were stained with ethidium bromide (0.2 mg/ml) for 1 h to show supercoiled, relaxed and nicked forms or kDNA and mini circle DNA and then photographed using a gel-imaging system. The quantity of supercoiled form in each lane was determined by densitometry and normalized to the blank group. The normalized quantity of the supercoiled form representing relative activity of the enzyme at the drug concentration was plotted to obtain an activity-dosage response curve. Median inhibition concentration (IC_{50}) of GolF toward each enzyme was interpolated from the activity–dose response curve.

Cell lines and culture conditions

Human hepatocellular carcinoma HepG2, Hep3B, and Huh7 cells as well as normal lung fibroblast MRC5 cells were cultivated in Dulbecco's modified Eagle's medium (DMEM). Chronic myelogenous leukemia K562 cells were cultivated in RPMI-1641. Both media were supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, 0.1 mM non-essential amino acids, and 2 mM L-glutamine. All cell lines were cultured at 37 °C in a humid atmosphere of 5% CO_2. Human peripheral blood mononuclear cells (PBMC) were isolated from sodium heparin venous blood obtained from healthy volunteers. Whole blood was centrifuged and the upper plasma layer was removed. PBMC were separated by centrifugation through a Ficoll-Hypaque gradient and then harvested from the interface of the gradient. The residual erythrocytes were removed by hypotonic lysis. The freshly isolated PBMC were cultured in RPMI-1640 medium containing supplements as described above.

Effects of GolF on the growth of cell lines

The cells were plated in 96-well plates and cultured for 18 h before drug additions. After cultivation in the presence of GolF for 24–96 h, cell viability was determined by Abacus cell proliferation (ACP) assay kit (BD Clontech, Palo Alto, CA, USA), which measured cellular acid phosphatase activity. Cell viability as function of absorbance at 410 nm was plotted versus dosage of drug to obtain growth inhibition curves and IC_{50}. Trypan blue exclusion was also used to evaluate drug effect (Lin et al., 2003; Li et al., 2005).

Assay for cellular DNA synthesis

The cells (1×10^4 cells/180 μl medium) were plated in 96-well plates with different concentrations of GolF or 0.5%