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6-Shogaol induces cell cycle arrest and apoptosis in human hepatoma cells through pleiotropic mechanisms

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ABSTRACT

Shogaols are a group of the active constituents of ginger that have been identified to have various biological activities. The aim of the current study was to investigate the antitumor activity of 6-shogaol in hepatocellular carcinoma (HCC) and the possible involvement of reactive oxygen species as a putative mechanism of action. HCC cell lines, HepG2 and Huh-7, were used to study the in vitro anti-cancer activity of 6-shogaol via the application of various molecular biology techniques. Results showed that 6-shogaol effectively inhibited the cell viability, caused cell cycle arrest at G2/M phase and induced apoptosis in HCC cells as indicated by MTT assay, DAPI nuclear staining, annexin V assay, cell cycle analysis, and activation of caspase-3. Western blot analysis revealed the ability of 6-shogaol to target cancer survival signaling pathways mediated by mitogen-activated protein kinase (MAPK), 5′ AMP-activated protein kinase (AMPK) and Akt. In addition, 6-Shogaol induced alteration of cyclin proteins expression and caused cleavage of protein kinase C delta. Furthermore, 6-Shogaol was able to induce the production of reactive oxygen species and endoplasmic reticulum (ER) stress-associated proteins and the consequent activation of autophagy in HepG2 cells. Taken together, the current study highlights evidences that 6-shogaol induces apoptosis, modulates cyclins expression and targets cancer survival signaling pathways in HCC cell lines, at least in part, via the production of reactive oxygen species. These findings support 6-shogaol's clinical promise as a potential candidate for HCC therapy.

1. Introduction

Ginger, the rhizome of *Zingiber officinale*, is extensively used in cooking worldwide and it is used as a traditional medicinal herb with antioxidant, anti-inflammatory and anticancer properties (Pan et al., 2008a; Park et al., 1998). 6-Shogaol is one of the phenolic alkanones isolated from rhizomes of ginger that exhibits significant antiproliferative activity in colon, liver, pancreatic, prostate, gastric, leukemia and lung cancers (Hung et al., 2009; Ishiguro et al., 2007; Lee and Surh, 1998; Pan et al., 2008b; Weng et al., 2010a). In human hepatoma, Mahlavu cells, 6-shogaol induces cell death through oxidative stress-mediated caspase activation (Chen et al., 2007). 6-Shogaol was reported to induce apoptosis in human colorectal carcinoma cells via reactive oxygen species production, caspase activation, and DNA damage-inducible gene 153 (GADD 153) expression (Hu et al., 2012). GADD 153 is induced in response to any stress that results in cell growth arrest or cell death, including DNA damage (Oh-Hashi et al., 2001), oxidative stress (Ikeyama et al., 2003), chemotherapeutic agents (Kim et al., 2002), and endoplasmic reticulum stress (ER stress) (Wang et al., 1996). In addition, 6-shogaol induces autophagy in human non-small cell lung cancer A549 cells by inhibiting the Akt/mTOR pathway (Hung et al., 2009). ER stress triggers complex signaling pathways known as the unfolded protein response (UPR) and the ER-overloading response pathway (EOR). UPR signaling in mammalian cells is mainly mediated through three pathway branches: activating transcription factor 6 (ATF6), inositol-requiring enzyme-

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1 (IRE1) and protein kinase-like endoplasmic reticulum kinase (PERK). In addition, ER stress leads to the accumulation of reactive oxygen species via the UPR-regulated oxidative protein folding machinery in the ER and mitochondria which usually results in cell death (Haynes et al., 2004).

Cyclins are key regulators of the mammalian cell cycle, functioning primarily in concert with their catalytic partners, the cyclin-dependent kinases (Cdks). The overexpression of cyclin proteins, such as cyclin D and cyclin E, has been linked to human cancers (Gillett et al., 1994; Keyomarsi et al., 1995). Therefore, the ability of these cyclins to activate cyclin-dependent kinases (CDKs) is the most extensively documented mechanism for their oncogenic actions and provides an attractive therapeutic target.

Hepatocellular carcinoma (HCC) is one of the most common causes of cancer morbidity and mortality in Asia and Africa. Hepatocellular cancer occurs both sporadically and in relation to chronic viral infection (Kao and Chen, 2005), environmental exposure (Chen et al., 1996), extensive alcohol intake (Thorgeirsson and Grisham, 2002), transgenic oncogenes (Feitelson et al., 2002; Lee et al., 2005; Suzuki et al., 1994) and other causes of hepatic cirrhosis. HCC usually has poor prognosis due to resistance to conventional chemotherapy and limited efficacy of radiotherapy (Avila et al., 2006).

While there are many studies reporting the activity of 6-shogaol as an anticancer agent, the exact mechanism of 6-shogaol-induced cell death is still unclear. This study investigated the efficacy of 6-shogaol and the possible mechanisms underlying its antitumor activity in HCC. It aimed at evaluating the relationship and interplay between reactive oxygen species production and cell signaling pathways modulated by 6-shogaol.

2. Materials and methods

2.1. Cell culture and chemicals

Huh-7 and HepG2 cell lines were obtained from the American Type Culture Collection (ATCC). Normal human hepatocytes, NeHepLxHT cells, were obtained from Dr. Lily Hui-Ching Wang. Cells were maintained at 37 °C in a 5% CO_2 incubator in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Ground Island, NY, USA). The culture media were replaced every 2 days. ECL Western blot detection system was purchased from Millipore (Billerica, MA, USA). Antibodies against various proteins were obtained from the following sources: PKCδ, Akt, p-Thr308-Akt, β-actin (Santa Cruz Biotechnology); cyclin A, cyclin B1, cyclin D1, cyclin E1, cyclin H, ERK, p-ERK, p38, p-p38, JNK, p-JNK, and PARP (Cell Signaling); LC3 II (sigma); GRP78 (Transduction Laboratories); ATF-6α, ATF-6β, Ire1α, Ire1β, eIF2α, and p-eIF2α (Genetex). Anti-rabbit IgG-horseradish peroxidase (HRP) conjugates, and rabbit anti-mouse IgG-HRP conjugates antibodies were purchased from Cell Signaling (Beverly, MA, USA). 6-Shogaol, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) and N-acetylcysteine were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Cell viability assay

Cell viability was assessed by MTT assay in six replicates as mentioned before (Omar et al., 2013). Briefly, HepG2 and Huh-7 cells were seeded at 5×10^3 per well in 96-well flat-bottomed plates and incubated in 10% FBS-supplemented DMEM for 24 h. Cells were treated with 6-shogaol at the indicated concentrations. Controls received vehicle (DMSO) at a concentration equal to that used in drug-treated cells. After 24 h, the drug-containing medium was replaced with 40 μl of 10% FBS-supplemented DMEM containing 0.5 mg/ml MTT and cells were incubated in the CO_2 incubator at 37 °C for 4 h. After removing the medium, the reduced MTT was solubilized in DMSO (100 μl per well) of, and the absorbance of 100 μl aliquots from each well was measured at 570 nm.

2.3. Cell cycle analysis

To determine the cell-cycle distribution, 5×10^5 cells seeded in 6-cm dishes were treated with various concentrations of 6-shogaol for 12 h. After incubation, the supernatant was removed, and the cells were then fixed in 70% ethanol/PBS, pelleted, and resuspended in a buffer containing RNase A and propidium iodide. Cell-cycle distribution was determined by flow cytometry, and the percentages of cells were determined using the FlowJo software (FLOWJO, LCC, Ashland, OR, USA).

2.4. Colony formation assay

For colony formation, Huh-7 and HepG2 cells were seeded at 1000 per well in 6-well flat-bottomed plates and incubated in 10% FBS-supplemented DMEM for 24 h. Cells were then treated with 6-shogaol at the indicated concentrations for 24 h. The culture medium was replenished, and cells were maintained at 37 °C for 14 days with medium change every other day. Grown colonies were fixed with 3.7% formaldehyde and stained with crystal violet. The number of cell colonies was determined in each well.

2.5. Analysis of caspase-3 activity

Caspase-3 activity was determined using PE active caspase-3 apoptosis kit (BD Pharmingen). Briefly, HepG2 (5×10^5) cells seeded in 6-cm dishes were subjected to different drug treatments for 24 h and then the cells were re-suspended in 0.5 ml Cytofix/Cytoperm solution for 20 min on ice. Cells were then incubated in 100 μl of Perm/Wash buffer containing 20 μl caspase-3 antibodies for 30 min at room temperature. Each sample was then washed with 400 μl Perm/Wash buffer, and caspase-3 activity signals were analyzed by flow cytometry.

2.6. Annexin V/propidium iodide assay

For the assessment of apoptosis, both floating and adherent cells were collected and analyzed. Briefly, 5×10^5 cells per dish were plated onto 6-cm dishes and incubated at 37 °C for 16 h. The cells were treated with DMSO or 10, 25 and 50 μM 6-shogaol for 12 h. After the treatment, the cells were washed twice with cold PBS and collected by trypsinization. After centrifugation at 400*g* for 5 min at room temperature, the cells were stained with Annexin V and propidium iodide (1 μg/ml). The cell apoptosis distribution was determined on a FACScort flow cytometer and analyzed by ModFitLT V3.0 and flowjo software program.

2.7. DAPI staining

The morphological changes in the nuclear chromatin of the cells undergoing apoptosis were detected by staining with DAPI as mentioned before (Weng et al., 2010b). Briefly, cells were treated with 6-shogaol alone or a combination of 6-shogaol with N-acetyl cysteine or salubrinal for 24 h, then cells were fixed in 3% paraformaldehyde for 15 min, and stained with DAPI for 2 min. Apoptotic nuclei were identified by the reduced nuclear size, condensed chromatin gathered at the periphery of the nuclear membrane or a totally fragmented morphology of nuclear bodies.