Anticancer effects of O-desmethylangolensin are mediated through cell cycle arrest at the G2/M phase and mitochondrial-dependent apoptosis in Hep3B human hepatocellular carcinoma cells

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Abstract. In the present study, in order to investigate the anticancer effects of O-desmethylangolensin (O-DMA) on human hepatocellular carcinoma Hep3B cells, we first examined the antiproliferative effect of O-DMA. When Hep3B cells were treated with O-DMA at various concentrations (5-200 µM) for 24, 48 or 72 h, cell proliferation decreased significantly in a dose- and time-dependent manner. Moreover, O-DMA exposure at the IC50 concentration for 72 h arrested cells at the G2/M phase, which was accompanied by a reduction in CDK1, and an increase in cyclin A and B. Under the same conditions, O-DMA significantly increased the number of sub-G1 phase cells. Additionally, an Annexin V assay revealed that exposure to O-DMA affected the rate of cell apoptosis. O-DMA caused the downregulation of Bcl-2 and upregulation of Bax, which led to cytochrome c release from the mitochondria and activation of caspase-3. Taken together, these data suggest that O-DMA exhibits anticancer activity by arresting the cell cycle at G2/M phase and causing mitochondrial-dependent apoptosis in Hep3B cells.

Introduction

Isoflavones belong to the flavonoids and exist in nature in commonly consumed plants. They have been suggested to possess antioxidant, anticancer and anti-inflammatory activities. Numerous reports have supported this concept, and isoflavones are generally accepted to have the potential to benefit human health.

Daidzein, which occurs in nature as daidzin (its β-glycoside form), is one of the main components of isoflavones. Gastrointestinal bacteria in humans metabolize daidzein to the reduced [equol and O-desmethylangolensin (O-DMA)] and oxidized (3’,4’,7-trihydroxyisoflavone and 4’,6,7-trihydroxyisoflavone) forms (1,2). Researchers have speculated that these metabolites play an important role in the biological mechanism of daidzein (3-5).

Studies have reported that daidzein inhibits the growth of various types of cancer cells such as breast (6,7), ovarian (8,9), prostate (10) and hepatocellular carcinoma (HCC) (11,12). In addition, daidzein has been shown to induce apoptosis by modulating molecular signaling pathways such as DNA damage-signaling (10), cell cycle regulation (6,10), receptor-mediated signaling (13-15) and the mitochondrial apoptotic pathway (7).

Nevertheless, no study has investigated the effect of the metabolite O-DMA on HCC, which is one of most common malignant tumors diagnosed worldwide. Therefore, we investigated the anticancer effects of O-DMA on cell cycle arrest and apoptosis induction in human HCC Hep3B cells.

Materials and methods

Cell culture. Human HCC Hep3B cells were purchased from the Korean Cell Line Bank (KCLB), Korea. Cells were routinely maintained in MEM (Sigma-Aldrich, USA), supplemented with 10% FBS and antibiotics (50 U/ml penicillin and 50 µg/ml streptomycin; Sigma) at 37˚C in a humidified atmosphere containing 5% CO2. In the cell proliferation experiment, cells were treated with different O-DMA concentrations (ranging from 5 to 200 µM) and then incubated for varying time periods (24, 48 and 72 h). In the cell cycle analysis and apoptosis assay, cells were treated with O-DMA at an IC50 concentration for 72 h.

Synthesis of O-DMA. O-DMA synthesis is illustrated in Fig. 1. Ethyl 2-((p-methoxyphenyl)propionate 2 was prepared by gentle heating of a mixture solution of p-methoxypropiophenone 1, 70% perchloric acid and lead(IV) acetate in triethyl orthoformate for 2 h at 50˚C in an 87% yield (Scheme 1). Compound 2 was hydrolyzed under basic conditions by the treatment of 0.5 N-KOH in CH3OH/H2O for 24 h at room temperature to afford 2-(p-methoxyphenyl)propionic acid 3 in a 93% yield after acidic work-up. The reaction of 3 with di-2-pyridyl carbonate in the presence of 0.02 equiv
of 4-dimethylaminopyridine (4-DMAP) in dichloromethane proceeded well for 3 h at room temperature to afford 2-pyridyl 2-(p-methoxyphenyl)propionate 4 in an 81% yield. The acyl substitution of 4 by 2,4-dimethoxyphenylmagnesium bromide in THF proceeded rapidly at 0˚C via 6-membered chelate to give 2,4,4’-trimethoxy-α-methyldesoxybenzoin 5 in a 95% yield after acidic hydrolysis. Compound 5 was demethylated by the treatment of 4 equiv of boron tribromide in dichloromethane for 48 h at room temperature to afford O-DMA 6 in a 90% yields after aqueous work-up. O-DMA was dissolved in dimethyl sulfoxide (DMSO) (final concentration 0.1% in medium) and kept in a refrigerator.

**MTT assay.** Hep3B cells were plated at a density of 1x10^5 cells/well in a 96-well tissue culture plate (Corning, NY, USA), and incubated at 37˚C for 24 h. Plated cells were treated with the indicated concentrations of O-DMA for 24, 48 and 72 h. After treatment, plated cell were incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 0.5 mg/ml final concentration; Sigma-Aldrich) for 4 h at 37˚C. After discarding all medium from the plates, 100 µl of DMSO was added to each well. The plates were placed for 5 min at room temperature with shaking, so that complete dissolution of formazan was achieved. The absorbance of the MTT formazan was determined at 540 nm by a UV spectrophotometric plate reader (EMax; Molecular Devices). The value of IC_{50} (i.e., the concentration of the extract required to inhibit cancer cell growth by 50% of the control level) was estimated from the plot. Control cells were treated with only the compound solvent.

**Cell cycle distribution.** Cell cycle distribution and apoptosis were determined by FACS analysis using propidium iodide (PI) staining to measure DNA content. Hep3B cells were plated at a density of 5x10^5 cells/well in a 6-well tissue culture plate (Corning), and incubated at 37˚C for 24 h. Plated cells were treated with an IC_{50} concentration of O-DMA for 72 h. Cells were then harvested, washed with cold PBS and processed for cell cycle analysis. Briefly, the cells were fixed in absolute ethanol and stored at -20˚C for later analysis. The fixed cells were centrifuged at 1,000 rpm and washed with cold PBS twice. RNase A (20 µg/ml final concentration) and PI staining solution (50 µg/ml final concentration) were added to the cells and incubated for 30 min at 37˚C in the dark. The cells were analyzed using a FACS Calibur instrument (BD Biosciences, San Jose, CA, USA) equipped with CellQuest 3.3 software.

**Detection of apoptosis.** Cells were treated and harvested similarly as mentioned in the preceding section. Phosphatidylserine is a biomarker of apoptosis, which is located on the cytoplasmic surface of the cell membrane. Phosphatidylserine exposure on the outer leaflet was detected using the Annexin V-FITC Apoptosis Detection kit (Calbiochem; EMD Chemicals Inc., Darmstadt, Germany). Annexin V and PI solution were added to the cell preparations, and incubation was carried out for 25 min in the dark. Binding buffer (400 µl) was then added to each tube, and the samples were analyzed by flow cytometry.

**Cytochrome c release assay.** Release of cytochrome c from the mitochondria to the cytosol was measured by immunoblotting assay. To detect the cytochrome c release, the cell lysates were centrifuged at 100,000 rpm for 30 min at 4˚C in order to obtain the supernatant (cytosolic fraction) and the pellet (fraction that contains the mitochondria). Proteins (25 µg/well) were separated on a 12% SDS-polyacrylamide gel and analyzed by immunoblot assay using anti-cytochrome c antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA).

**Immunoblot assay.** Cells were lysed in RIPA buffer (1% NP-40, 150 mM NaCl, 0.05% DOC, 1% SDS, 50 mM Tris; pH 7.5) containing protease inhibitor at 4˚C for 1 h. The supernatant was separated by centrifugation, and protein concentration was determined using the Bradford protein assay kit II (Bio-Rad). Proteins (25 µg/well) were separated on a 10% SDS-polyacrylamide gel. Proteins were transferred onto 0.45-µm nitrocellulose membranes. The membranes were blocked with a 1% BSA solution for 3 h and washed twice with PBS containing 0.2%
Tween-20, and incubated with the primary antibody at 4˚C overnight. Antibodies against CDK1, cyclin A, cyclin B, Bcl-2, Bax, precursor caspase-3, cleaved caspase-3, cytochrome c and β-actin (all from Santa Cruz Biotechnology, Inc.) were used to probe the separate membranes. On the next day, the immunoreaction was continued using the secondary goat anti-rabbit horseradish peroxidase-conjugated antibody after washing for 2 h at room temperature. The specific protein bands were detected using the Opti-4CN substrate kit (Bio-Rad).

Statistical analyses. All values are expressed as the means ± SD. Data were analyzed by unpaired Student’s t-test or one-way analysis of variance followed by Dunnett’s multiple comparison test (SigmaStat; Jandel, San Rafael, CA, USA). For all comparisons, differences were considered statistically significant at P<0.05.

Results

Effect of O-DMA on cell proliferation of Hep3B cells. The antiproliferative effect of O-DMA was determined using an MTT assay in human HCC Hep3B cells exposed to O-DMA at various concentrations (5, 10, 25, 50, 75, 100, 125, 150 and 200 µM) for 24, 48 and 72 h (Fig. 2). O-DMA significantly reduced cell proliferation in a dose- and time-dependent manner (P<0.05), but inhibition of the cell proliferation increased significantly after 72 h. The IC₅₀ values at 48 and 72 h were 135.02 and 106.14 µM, respectively. To compare O-DMA with the precursor daidzein, we investigated the anti-proliferative activity of daidzein under the same conditions. We found that daidzein had a higher IC₅₀ (4.7-fold higher compared to O-DMA) at 24 h. The IC₅₀ values at 48 and 72 h were 243.2 and 258.2 µM, respectively.

Figure 2. Antiproliferative activity of (A) O-DMA and (B) daidzein. Hep3B cells were exposed to either vehicle or O-DMA (5-200 µM) and incubated for 24, 48 and 72 h. All data are reported as the percentage of change in comparison with the vehicle-only group, which was arbitrarily assigned 100% viability. *P<0.005, significantly different from the vehicle-only group (0.1% DMSO in medium).

Effect of O-DMA on the cell cycle distribution of Hep3B cells. Based on these results, the IC₅₀ concentration after 72 h exposure to O-DMA was used to verify cell cycle arrest. After exposure of Hep3B cells to O-DMA, the proportion of G1 phase cells was decreased by 15.9%, and the proportion of S and G2/M-phase cells was significantly increased by 40.7 and 45.4%, respectively, compared with control cells (Fig. 3A, P<0.05). With respect to G2/M-related proteins, reduced CDK1 expression and slightly increased cyclin A and B was noted in the Hep3B cells following O-DMA treatment (Fig. 3B).

Under the same conditions, exposure to O-DMA significantly shifted the cell populations (Fig. 4A). The percentages of early and late apoptotic cells were 27.4 and 6.1%, respectively, in Hep3B cells exposed to O-DMA. Moreover, small DNA fragments in the sub-G1 phase increased by 18.5% after O-DMA treatment (Fig. 3A).

Figure 3. G/M phase arrest by O-DMA and its related gene expression. (A) Hep3B cells were exposed to either vehicle (0.1% DMSO in medium) or O-DMA at IC₅₀ for 72 h. Cell cycle distribution was determined by FACS analysis using PI staining to measure DNA content. Data are expressed as the means ± SD from 4 independent experiments. Values are expressed as the percentage of the cell population in the G1, S and G1/M phase of the cell cycle. (B) Expression of G/M phase-related genes was determined by immunoblot assay. *P<0.005, significantly different from the vehicle-only group (0.1% DMSO in medium).

Effect of O-DMA on the expression of apoptosis-related genes in Hep3B cells. O-DMA had an effect on Bcl-2, Bax and caspase-3 expression, as well as cytochrome c release (Fig. 4B and C). Apoptosis induced by O-DAM decreased Bcl-2 and increased Bax expression levels. Moreover, the
increase in cytochrome c release induced by O-DMA suggests that O-DAM triggers caspase-activated apoptosis. The caspase-3 cleavage product was also observed after O-DMA treatment.

Discussion

HCC is a global health problem. According to GLOBOCAN 2008 estimates (16), HCC is the fifth and seventh most common cancer, and the second and sixth most fatal cancer in men and women, respectively (17).

In the present study, we first examined the antiproliferative effect of O-DMA on human HCC Hep3B cells to investigate its anticancer activity. O-DMA demonstrated antiproliferative activity in a dose- and time-dependent manner, and its IC_{50} value was significantly lower than that of its precursor, daidzein. Moreover, daidzein stimulated cell growth at concentrations lower than 50 µM after 24 h, consistent with previous reports stating that daidzein may have biphasic effects on the growth of several types of cancer cells (18,19). This observation is explained by the hypothesis that phytoestrogens, which are plant-derived, are structurally similar to estradiol (17β-estradiol) and bind to estrogen receptors (ERs) such as daidzein and its metabolite equol. This can stimulate or inhibit cell proliferation by functioning as an estrogen agonist or antagonist (20-22). Alternatively, O-DMA has a very weak affinity to the ERs (23-25), and O-DMA was shown to reduce the proliferation of breast cancer MCF-7 cells in an estrogen-independent manner (18).

Although O-DMA and daidzein have similar effects at higher concentrations after 72 h, the fact that daidzein stimulates cell proliferation under certain conditions (as an estrogenic) suggests that O-DMA may be a good anticancer agent. However, O-DMA exposure at an IC_{50} concentration for 72 h caused cell cycle arrest at the G2/M phase and subsequent apoptosis, which may be an issue for its use in cancer chemotherapy.

O-DMA reduces CDK1 (Cdc2) and increases cyclin A and B, which may explain the G2/M phase arrest. G2/M transition controls the onset of mitosis and is regulated by cyclin A (in the earlier stages) and the CDK1-cyclin B complex (at later stages) (26). CDK is often mutated, deleted or silenced in cancer, leading to the search for small-molecule CDK inhibitors in cancer therapy (27-29).

O-DMA treatment induced apoptosis in Hep3 cells, confirming the sub-G1 peak and the shift in cell populations shown by the Annexin V assay. Note that the main mechanism of apoptosis induction by O-DMA may not involve the p53 pathway since Hep3B is a p53-deficient cell line. In our preliminary experiments, O-DMA at concentrations over 100 µM significantly inhibited approximately 30% of cell viability of HepG2 cells. However, at concentrations lower than 100 µM, cell growth was not affected by the addition of O-DMA (data not shown). Generally, HepG2 cells are highly susceptible to chemical agents and drugs, while Hep3B cells are more resistance (30,31). Therefore, the significant anticancer effect of O-DAM in Hep3B cells supports its use as a cancer drug candidate.

In addition, expression of Bcl-2 decreased while Bax increased in the Hep3B cells after exposure to O-DMA. Members of the Bcl-2 family of proteins are critical regulators of the apoptotic pathway, controlling mitochondrial permeability and cytochrome c expression (32,33). These proteins include major anti-apoptotic family members, Bcl-2 and Bcl-xL, and the major pro-apoptotic proteins, Bax and Bak. This is consistent with the anticancer mechanism of daidzein involving the modulation of Bcl and Bax in several cancer cell lines such as breast, prostate and head and neck (7,34,35).

Additionally, O-DMA induced cytochrome c release and decreased the expression of a caspase-3 precursor. Release of
cytochrome c from the mitochondria to the cytoplasm is a key step in the initiation of mitochondrial-dependent apoptosis (36). As a downstream product of cytochrome c, caspases are critical mediators of the principal factors found in apoptotic cells. Among them, caspase-3 is a frequently activated death protease, catalyzing the specific cleavage of many cellular proteins (37,38).

In conclusion, our results demonstrated that O-DMA inhibited cancer cell growth via cell cycle arrest and apoptosis in HCC Hep3B cells. O-DMA-treated cells were arrested at the G2/M phase, which was accompanied by a reduction in CDK1. Downregulation of Bcl-2 and upregulation of Bax led to cytochrome c release from the mitochondria and activation of caspase-3. These findings indicate that O-DMA is a promising anticancer drug candidate.

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