Chemico-Biological Interactions 188 (2010) 161–170

Contents lists available at ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

1-(3′,4′,5′-Trimethoxyphenyl)-3-(3″,4″-dimethoxy-2″-hydroxyphenyl)-propane with microtubule-depolymerizing ability induces G2/M phase arrest and apoptosis in HepG2 cells

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ARTICLE INFO

Article history:
Received 12 May 2010
Received in revised form 15 July 2010
Accepted 22 July 2010
Available online 30 July 2010

Keywords:
1,3-Diarylpropanes
G2/M phase arrest
Apoptosis
Microtubules
Mitochondria
Reactive oxygen species

ABSTRACT

1-(3′,4′,5′-Trimethoxyphenyl)-3-(3″,4″-dimethoxy-2″-hydroxyphenyl)-propane (DP), a novel synthesized 1,3-diarylpropanes compound, showed growth inhibitory effect on human hepatoma HepG2 cells in a concentration-dependent manner. The growth inhibitory effect of DP on HepG2 cells was associated with microtubule depolymerization, G2/M phase arrest and apoptosis induction. The G2/M phase arrest induced by DP resulted from its microtubule-depolymerizing ability, and DP-treated HepG2 cells finally underwent caspase-dependent apoptosis. DP increased the levels of death receptor 4 (DR4), death receptor 5 (DR5) and pro-apoptotic protein Bax, but decreased the levels of anti-apoptotic protein Bcl-2. Meanwhile, the decrease in the mitochondrial membrane potential (MMP) and the release of cytochrome *c* from mitochondria were observed in DP-treated HepG2 cells. DP increased the levels of reactive oxygen species (ROS) in HepG2 cells, and antioxidant N-acetylcysteine (NAC) completely blocked DP-induced ROS accumulation and the disruption of the balance between Bax and Bcl-2 proteins, and effectively blocked the decreased MMP and apoptosis, but had no effect on the activation of caspase-8 and the up-regulations of DR4 and DR5 induced by DP. These results suggest that DP induces G2/M phase arrest through interruption of microtubule network followed by the death receptor- and ROS-mediated apoptosis in HepG2 cells.

1. Introduction

Cancer is a leading cause of death worldwide and its incidence is still rising in many countries. The malignant properties of cancer, especially metastasis, often put it beyond the potential for surgical extirpation or local ablation by radiotherapy. The limitations of these two kinds of treatments make chemotherapy – a systemic drug based approach – irreplaceable [1]. In the chemotherapy field, the natural products have been considered as the main source of chemotherapeutics and are likely to provide more lead structures with enhanced biological activities [2].

Microtubules are dynamic polymers of α- and β-tubulin subunits which play a central role in a variety of fundamental cell functions, including cell shape control, intracellular motility and cell division regulation. Disruption of microtubules can engage the mitotic spindle checkpoint, arrest cell cycle progression at mitosis and eventually lead to apoptosis [3]. Their critical role in mitosis makes them an attractive target for anti-cancer drugs. A large number of chemically diverse compounds are able to bind tubulin or microtubules (generally bind to one of the two classes of sites on tubulin, the vinca domain and paclitaxel site) and inhibit the proliferation by interfering the mitotic spindle. Some of these compounds, such as vinca alkaloids, colchicinoids and combretastatin-A4 (CA-4), inhibit microtubule polymerization, whereas others, such as taxanes and epothilones, stabilize microtubules [4,5]. Although these compounds exert opposite effects on microtubules, both types share the common properties of suppressing microtubule dynamics and thereby microtubule function [6].

1,3-Diarylpropanes are a set of natural products present in plants such as *Terminalia fagifolia* Mart [7], *Viscum articulatum* [8] *Phacellaria compressa* Benth [9] and *Dioscorea composita* Hemsl [10], some of which exhibit anti-cancer activity. For example, two natural 1,3-diarylpropanes, 1-(4′-hydroxy-2′-methoxyphenyl)-3-(3″-methoxy-4″-hydroxyphenyl)-propane and 1-(2′-hydroxy-4′,6′-dimethoxyphenyl)-3-(3″-methoxy-4″-hydroxyphenyl)-propane showed *in vitro* cytotoxic activity against two human cancer cell lines (Hep2 larynx carcinoma and H292 lung mucoepidermoid carcinoma) [7], but the mechanisms of their

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doi:10.1016/j.cbi.2010.07.021

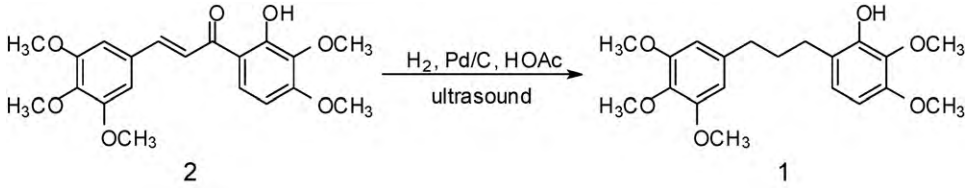

Fig. 1. Synthesis of DP.

actions have never been investigated. In our effort to search for new anti-cancer agents using natural 1,3-diarylpropanes as lead compounds, 1-(3′,4′,5′-trimethoxyphenyl)-3-(3″,4″-dimethoxy-2″-hydroxyphenyl)-propane (DP) was synthesized and showed outstanding anti-proliferative activity against several tumor cell lines. The aim of the present study was to investigate the growth inhibitory effect and the molecular mechanisms of DP in human hepatoma HepG2 cells.

2. Materials and methods

2.1. Reagents

DP as a white solid was synthesized by treating chalcone (2) (which was prepared via Claisen condensation of 1-(2-hydroxy-3,4-dimethoxyphenyl)-ethanone with 3,4,5-trimethoxybenzaldehyde [11]) under H_2 atmosphere using Pd/C (10%) as catalyst in HOAc in an ultrasonic cleaner with frequency of 25 kHz and a nominal power of 400 W at 20–23 °C for 2 h in 79% yield (Fig. 1). DP was dissolved in dimethyl sulfoxide (DMSO) and kept frozen at −20 °C. The final DMSO concentration in the medium did not exceed 0.1% (v/v). At this concentration, DMSO had no effect on cell growth or viability of HepG2 cells used in this study for up to 96 h.

N-acetylcysteine (NAC), propidium iodide (PI), acridine orange (AO) and ethidium bromide (EB) were purchased from Sigma Chemical Co. (St. Louis, MO). Rhodamine 123 and 5,6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) were purchased from Molecular Probes (Eugene, OR). The inhibitors of caspase-3 (Z-DEVD-FMK), caspase-8 (Z-IETD-FMK) and caspase-9 (Z-LEHD-FMK) were purchased from Calbiochem (San Diego, CA). The primary antibodies to Cdk1, cyclin B1, poly (ADP-ribose) polymerase (PARP), caspase-3, caspase-8, caspase-9, cytochrome *c*, Bcl-2, Bax, Bid, α-tubulin antibody and horseradish peroxidase-labeled secondary anti-mouse and anti-rabbit antibodies were purchased from Cell Signaling Technology (Beverly, MA). The primary antibodies to 4-hydroxynonenal (4-HNE) and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The primary antibodies to anti-DR4 and anti-DR5 and FITC conjugated secondary antibody were purchased from Chemicon International (Temecula, CA).

2.2. Cell culture

HepG2 cells were cultured in RPMI 1640 complete medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 1 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified 5% CO_2 incubator.

2.3. Cell growth inhibition assay

The growth inhibitory effect of DP on HepG2 cells was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. HepG2 cells were seeded in 96-well plates (5×10^3 cells per well in 100 μl). After 12 h of incubation, the cells were treated with different concentrations (0.25–4.0 μM) of DP. As a control group, DMSO (end-concentration of 0.1%) was added. After 72 or 96 h, MTT solution [5.0 mg/ml in phosphate-buffered saline (PBS)] was added (20 μl/well), and the plates were incubated for another 4 h at 37 °C. The purple formazan crystals were dissolved in 100 μl DMSO and the plates were read on an ELISA plate reader at 570 nm. The cell viability was calculated as the ratio of the absorbance of the treated cells to the absorbance of the control groups. The drug concentration at which the compound inhibited half of the cell growth (IC_{50}) was calculated using the software of Statistical Product and Service Solutions. Assays were performed in triplicate in three independent experiments.

2.4. Flow cytometric analysis

Cell cycle distribution was detected using flow cytometry. HepG2 cells (1×10^6 cells) were incubated with various concentrations of DP or 0.1% DMSO for indicated times. After harvesting by trypsinisation, the cells were washed with PBS, and fixed in ice-cold 70% (v/v) ethanol. The fixed cells were harvested by centrifugation and resuspended in 500 μl of PBS containing 50 mg/ml RNase. After 30 min incubation at 37 °C, the cells were stained with 50 mg/ml PI at 4 °C in dark for 30 min, and flow cytometry was then performed on FACScan, with collection and analysis of data using CellQuest software.

2.5. AO/EB fluorescence staining

AO/EB fluorescence staining is used to visualize nuclear changes and apoptotic body formation that are characteristic of apoptosis. Cells are viewed under a fluorescence microscope and counted to quantify apoptosis [12]. HepG2 cells (3×10^4 cells) were plated with submerged cover slips in 6-well plates and incubated with various concentrations of DP or 0.1% DMSO for indicated times. Then the cells were washed with PBS twice and stained with 10 μl of the dye mixture, containing 100 mg/ml of AO and 100 mg/ml of EB in PBS. After staining, the cells were immediately visualized under a fluorescence microscope. EB-negative cells with nuclear shrinkage, blebbing and apoptotic bodies were observed and counted as apoptotic cells. The percentage of apoptotic cells was calculated after observing a total of 300 cells [13].

2.6. Agarose gel electrophoresis

DNA fragmentation was evaluated by agarose gel electrophoresis. HepG2 cells (1×10^6 cells) were incubated with various concentrations of DP or 0.1% DMSO for indicated times. After harvesting by trypsinisation, the cells were washed with PBS, and harvested by centrifugation. The pellets were resuspended in a lysis buffer (10 mM Tris at pH 7.4, 5 mM ethylenediamine tetraacetic acid (EDTA), 1% Triton X-100) for 20 min on ice. After centrifugation, supernatants were removed and treated with 20 mg/ml RNase at 37 °C for 1 h, and 0.1 mg/ml proteinase K for additional 1 h. DNA fragments were then extracted, separated on 2% agarose gel, and imaged after staining with EB.