Growth Inhibition and Cell Cycle Arrest in the G0/G1 by Schizandrin, a Dibenzocyclooctadiene Lignan Isolated from Schisandra chinensis, on T47D Human Breast Cancer Cells

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Schizandrin is one of the main dibenzocyclooctadiene lignans present in the fruit of Schisandra chinensis (Schisandraceae). Biological activities including hepatoprotective, antiviral and neuroprotective effects of schizandrin and other dibenzocyclooctadiene lignans have been reported previously. However, the antiproliferative effect of schizandrin against human cancer cells has been poorly determined to date. This study examined the antiproliferative effect of schizandrin in human breast cancer cells. Schizandrin exhibited growth inhibitory activities in cultured human breast cancer cells, and the effect was the more profound in estrogen receptor (ER)-positive T47D cells than in ER-negative MDA-MB-231 cells. When treated with the compound in T47D cells, schizandrin induced the accumulation of a cell population in the G0/G1 phase, which was further demonstrated by the induction of CDK inhibitors p21 and p27 and the inhibition of the expression of cell cycle checkpoint proteins including cyclin D1, cyclin A, CDK2 and CDK4. These results suggest that schizandrin inhibits cell proliferation through the induction of cell cycle arrest with modulating cell cycle-related proteins in human breast cancer cells. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: schizandrin; Schisandra chinensis; cell cycle arrest; T47D; breast cancer.

INTRODUCTION

Breast cancer is the most common cancer in women and known to be one of the five common cancers in the world. Surgery, chemotherapy, endocrine therapy and radiation therapy are generally used in the treatment of breast cancer, but several side effects and drug resistance are also frequent problems in the course of therapy (Bange et al., 2001). Therefore, it is necessary to discover novel agents that can be used for chemotherapy and/or adjuvant therapy for breast cancer.

Schizandrin is a lignan of the dibenzocyclooctadiene type which is found mainly in the fruit of Schisandra chinensis (Schisandraceae). Hancke et al., 1999. The fruit of S. chinensis has been used as an antiaging agent, a stimulant, a cough remedy and a tonic (Hancke et al., 1999). Pharmacological studies also suggest that the extracts or constituents, mainly dibenzocyclooctadiene lignans, of the fruit of S. chinensis possess hepatoprotective, antioxidant, antiviral, neuroprotective and cancer chemopreventive activities (Yasukawa et al., 1992; Chen et al., 1997; Hancke et al., 1999; Zhu et al., 1999; Kim et al., 2004). In addition, dibenzocyclooctadiene lignans such as gomisin A, schisandrin A, schisandrin B and schisantherin A reversed multidrug resistance or enhanced the cytotoxic effect of anticancer agents (Li et al., 2006; Wan et al., 2006). Schisantherin G and propinquanin E also showed cytotoxicity in human cancer cells (Xu et al., 2006).

These previous reports suggest various biological activities, especially cytotoxic and antitumor potential, of dibenzocyclooctadiene lignans. Schizandrin was reported previously to alleviate memory impairment and to inhibit allergic responses in animal models (Lee et al., 2007; Egashira et al., 2008). However, the inhibitory effect of schizandrin against the proliferation of human cancer cells has been poorly examined. This study was performed to investigate the antiproliferative effect and the mechanism of action of schizandrin in human breast cancer cells. The results suggest that schizandrin inhibits the proliferation of human breast cancer cells through cell cycle arrest with modulation of the expression of cell cycle-regulatory proteins.

MATERIALS AND METHODS

Chemicals. Trichloroacetic acid (TCA), sulforhodamine B, propidium iodide, RNase A and mouse monoclonal anti-β-actin antibody were purchased from Sigma-Aldrich (St Louis, MO, USA). Dulbecco’s modi-
fied Eagle medium (DMEM), RPMI 1640 medium, fetal bovine serum (FBS), trypsin-EDTA solution and antibiotics–antimycotics solution were from Invitrogen (Grand Island, NY, USA). Mouse monoclonal anti-p53 (DO-1) antibody, rabbit polyclonal anti-CDK2 (M2), anti-CDK4 (H-22), anti-CDK2 (17), anti-cyclin A (H-432), anti-cyclin B1 (H-433), anti-p21 (C-19), anti-27 (C-19) antibodies, horseradish peroxidase (HRP)-conjugated anti-mouse IgG and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse monoclonal anti-cyclin D1 and anti-cyclin E antibodies were purchased from BD Biosciences (San Jose, CA, USA). Schizandrin was isolated from the n-hexane extract of *S. chinensis* and identified by comparison of spectroscopic data with the previous report (Fig. 1) (Ikeya et al., 1979).

**Cell culture.** T47D (estrogen receptor (ER)-positive human breast carcinoma cells), MDA-MB-231 (ER-negative human breast adenocarcinoma cells) and MCF-10A (human breast epithelial cells) cells were obtained from the Korean Cell Line Bank (KCLB, Seoul, Korea). MDA-MB-231 and T47D cells were cultured in RPMI 1640 medium (T47D cells) and DMEM (MDA-MB-231 cells) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/mL penicillin, 100 µg/mL streptomycin and 250 ng/mL amphotericin B. MCF-10A cells were cultured in DMEM/F12 medium supplemented with 10% FBS, 100 units/mL penicillin, 100 µg/mL streptomycin, 2 ng/mL epidermal growth factor (EGF) and 4 µg/mL insulin. The cells were maintained at 37 ºC in a humidified atmosphere with 5% CO₂.

**Evaluation of antiproliferative potential.** MDA-MB-231, T47D and MCF-10A cells (5 × 10⁶ cells/mL) were treated with various concentrations of schizandrin for 3 days. The cell viability was determined with the sulforhodamine B (SRB) staining method as described previously (Lee et al., 2002). The result was expressed as a percentage, relative to solvent-treated control incubations, and the IC₅₀ values were calculated using non-linear regression analysis (percent survival versus concentration).

**Cell cycle analysis.** T47D cells were plated at a density of 1 × 10⁶ cells per 100 mm culture dish. After 24 h, the cells were washed with PBS and treated with various concentrations of schizandrin diluted in the fresh medium and further incubated for 24 h. Adherent and floating cells were collected and washed with PBS twice. The cells were fixed with 100% methanol overnight and incubated with a staining solution containing RNase A (50 µg/mL) and propidium iodide (50 µg/mL) in phosphate-buffered saline (PBS). The cellular DNA content was analysed by flow cytometry (FACSCalibur, BD Biosciences). At least 20000 cells were used for each analysis, and the results are displayed as histograms. Cell cycle distribution was analysed using the ModFit LT 2.0 program.

**Western blot analysis.** Cells (1 × 10⁶ cells/dish in a 100 mm dish) were treated with various concentrations of schizandrin for 24 h. Total cell lysates were obtained using boiling 2X sample loading buffer (250 mM Tris-HCl (pH 6.8), 4% SDS, 10% glycerol, 0.006% bromophenol blue, 2 mM sodium orthovanadate and 2% β-mercaptoethanol). The protein concentration of each lysate was determined by the BCA method. Lysates (30–50 µg) were subjected to 8–12% SDS-PAGE. Proteins were transferred onto PVDF membranes, and the membranes were incubated with blocking buffer (5% non-fat dry milk in PBS-0.1% Tween 20 (PBST)) for 1 h at room temperature. Membranes were then incubated with the indicated antibodies overnight at 4 ºC, and washed three times (each for 5 min) with PBST. Membranes were incubated with corresponding secondary antibodies for 1–2 h at room temperature. After washing three times (each for 5 min) with PBST, the proteins were detected using ECL detection reagent.

**Statistics.** Data were presented as the mean ± SD for the indicated number of independently performed experiments. Figure data are shown as one representative of at least three independent experiments.

**RESULTS AND DISCUSSION**

This study evaluated the effect of schizandrin on the proliferation of human breast cancer cells and further investigated the mechanism of action. First, the antiproliferative effect of schizandrin in breast cancer cells was tested. T47D (ER-positive) and MDA-MB-231 (ER-negative) cells were treated with various concentrations of schizandrin for 3 days, and the cell viability of each tested group was determined by the SRB staining method. As a result, schizandrin inhibited the cell proliferation of both human breast cancer cells in a concentration-dependent manner, but the effect was about twice more potent in ER-positive T47D cells than in ER-negative MDA-MB-231 cells with IC₅₀ values of 28.1 µm and 61.3 µm, respectively (Fig. 2). The IC₅₀ values of ellipticine used as a positive control were 3.2 µm in MDA-MB-231 cells and 3.1 µm in T47D cells. In addition, schizandrin did not inhibit cell growth in normal human breast epithelial cells (MCF-10A cells; Fig. 2(C)), suggesting that these antiproliferative effects of schizandrin might be relatively selective against cancer cells. According to this result, T47D cells were used for further studies.
To investigate the antiproliferative mechanism of schizandrin in T47D cells, the changes of cell cycle progression were analysed by flow cytometry, and the effect of schizandrin on the expression of cell cycle-regulatory proteins was determined by Western blot analysis. Cells treated with schizandrin moderately induced the accumulation of the cell population in the G0/G1 phase, and the distribution in the S phase was gradually decreased by schizandrin treatment in a concentration-dependent manner (Fig. 3). Cell cycle arrest in the G0/G1 phase was most pronounced in cells treated with 50 µM schizandrin. The cell population in the G2/M phase was slightly increased by treatment with 100 µM schizandrin, but this effect appeared to be less distinctive compared...
with schizandrin-mediated G0/G1 arrest at the same concentration. Therefore, it is suggested that schizandrin might generally exhibit G0/G1 phase cell cycle arrest in T47D cells. It is not clear, however, that schizandrin induces cell cycle arrest only at G0/G1 phase or biphasic arrest at G0/G1 and G2/M phases, because a change in the level of cell cycle seems to be not noticeable in these results. In order to clearly determine an effect on cell cycle progression, it would be necessary to synchronize the cell cycle of cultured cells in specific phases (for example, G0/G1 phase) and then treat the cells with schizandrin.

It is known that cell cycle progression is regulated by the sequential activation of the specific cyclin/cyclin-dependent kinase (CDK) complexes in each phase of the cell cycle (Golias et al., 2004). Upon stimulation by growth factor and other mitogenic signals, quiescent cells enter the cell cycle. The cyclin D/CDK4 complex promotes G1 phase progression toward S phase. CDK2 makes a complex with cyclin E in the late G1 to early S phase and with cyclin A during S phase. In addition, CDC2 (CDK1) is associated with cyclin B in the G2/M phase. The activity of cyclin/CDK complexes is negatively regulated by CDK inhibitors (CKIs) such as p21 and p27 (Sherr and Roberts, 1999). To investigate whether schizandrin affects the expression of these cell cycle-regulatory proteins, T47D cells were treated with various concentrations of schizandrin for 24 h, and the level of protein expression was examined by Western blot analysis. As depicted in Fig. 4, schizandrin dose-dependently suppressed the expression of cyclin D1 and CDK4, and the expression level of cyclin A, cyclin B1, CDK2, and CDC2 was also down-regulated in cells treated with 100 mM schizandrin. The level of p21 and p27 expression was also markedly up-regulated by treatment with schizandrin, especially the increase of p27 expression was concentration-dependent. It is known that p27 suppresses the kinase activity of cyclin E-cdk2 complex, which, in turn, blocks phosphorylation of pRB and inhibits cell cycle progression toward S phase (Sherr and McCormick, 2002). p21 is also known to be involved in cell cycle arrest in the G0/G1 and/or G2/M phases (Sherr and Roberts, 1999). Along with the modulation of the expression of cyclins and CDKs, up-regulation of p21 and p27 might be associated with schizandrin-mediated cell growth inhibition in T47D cells. The tumor suppressor p53 expression was not much changed in cells treated with schizandrin, presumably due to possessing mutated p53 in T47D cells (Nigro et al., 1989). Therefore, the increase of p21 expression by schizandrin appears to be p53-independent. Collectively, these results suggest that modulation of the expression of cell cycle-regulatory proteins might be related to the antiproliferative effect of schizandrin in T47D cells.

In summary, these findings indicate the antiproliferative effect of a natural dibenzocyclooctadiene lignan schizandrin in human breast cancer cells. Schizandrin showed effective inhibitory activities in both ER-positive breast cancer cells (T47D) and, to a lesser extent, ER-negative breast cancer cells (MDA-MB-231). Although the antiproliferative potency of schizandrin was lower than conventional chemotherapeutic agents, these results might reflect an additional biological activity and a therapeutic potential of schizandrin in the treatment of breast cancer.

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REFERENCES


