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Lignans extracted from *Vitex negundo* possess cytotoxic activity by G2/M phase cell cycle arrest and apoptosis induction

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ABSTRACT

Evn-50 is a lignan compounds mixture extracted from *Vitex negundo*, a widely used herb in traditional Chinese medicine. This study is aimed to define the spectrum of cytotoxic activity of EVn-50, and also to investigate mechanisms underlying the anticancer actions *via* assessing the influence on cell cycle using EVn-50, and the lignan compound VB1 purified from EVn-50. The cytotoxic effect of EVn-50 and VB1 was determined with SRB assay using a panel of cancer cell lines. Breast cancer cell line MDA-MB-435 and liver cancer cell line SMMC-7721 were selected for further evaluating the effect of EVn-50 or VB1 on cell cycle by flow cytometric analysis. Apoptosis exerted by EVn-50 or VB1 was measured by TUNEL assay and DAPI staining, and Western blot analysis was utilized to assess the influence on expression and phosphorylation of proteins which are closely related to cell cycle and apoptosis. EVn-50 possessed a broad spectrum of *in vitro* anticancer activity for those tested cancer cells, especially sensitive to MDA-MB-435, SKOV-3, BXPC-3, SMMC-7721, MCF-7, HO-8910, SGC-7901, BEL-7402, HCT-116, and 786-O, with the respective IC50 below 10 μg/ml. Treatment with EVn-50 or VB1 resulted in arresting the MDA-MB-435 and SMMC-7721 cells at G2/M phase, which was further supported by observations of increased phosphorylation of Histone 3 at Ser10, phosphorylation of Cdk1 at Tyr15, expression of cyclin B1, and decreased expression of Cdc25c. Moreover, we found that exposure of MDA-MB-435 cells to EVn-50 or VB1 caused obvious apoptosis of MDA-MB-435 cells. Our data show that EVn-50, lignan compounds extracted from *Vitex negundo*, possesses a broad spectrum cytotoxic effect *via* arresting cancer cells at G2/M phase cell cycle and subsequently inducing apoptosis.

Introduction

Lignans are widely present in fruits, cereals, soybeans, vegetables, *etc.*, with particular higher concentration in flaxseed and sesame seeds (Adlercreutz 2002). Once intaked, plant lignans are converted by intestinal bacterial to mammalian lignans such as enterolignans (ENL), enteroldiol (END), which are subsequently absorbed (Axelson and Setchell 1981; Borriello et al. 1985). The biological activity of plant lignans is supposed to be mediated by their metabolized mammalian products, ENL and END (Stattin et al. 2002). Many studies have shown that lignans possess various pharmacological effects, including antioxidant, antimicrobial, anti-inflammatory and immunosuppressive activities (Adlercreutz 2007). In addition to these pharmacological activities, emerging evidences including preclinical studies and clinical trials demonstrate that plant lignans may reduce the risk of cancers and also suppress cancer growth, especially for those hormone-dependent cancers (Adlercreutz 2002). As lignans are one of the major classes of phytoestrogens with an estrogen similar structure, it is not surprising that they are capable of binding to estrogen receptors and interfering with the cancer-promoting effects of estrogen, thus possessing breast cancer prevention efficacy (Mueller et al. 2004; Penttinen et al. 2007; Saarinen et al. 2010). Moreover, lignans have been also demonstrated to prevent other hormone-dependent cancer such as prostate cancer and ovarian cancer (Chen et al. 2007).

Vitex negundo, a widely used herb in China, has been shown to possess anti-inflammatory efficacy and used for treatment of cough, asthma, rheumatism, and arthritis in folk medicine in some areas in China. Zhou et al. have isolated a mixture of lignan compounds from seeds of *Vitex negundo*, which is named EVn-50. The total lignan compounds constitute 70% of EVn-50 (Zhou et al. 2009). Furthermore, fifteen lignan compounds have been purified from

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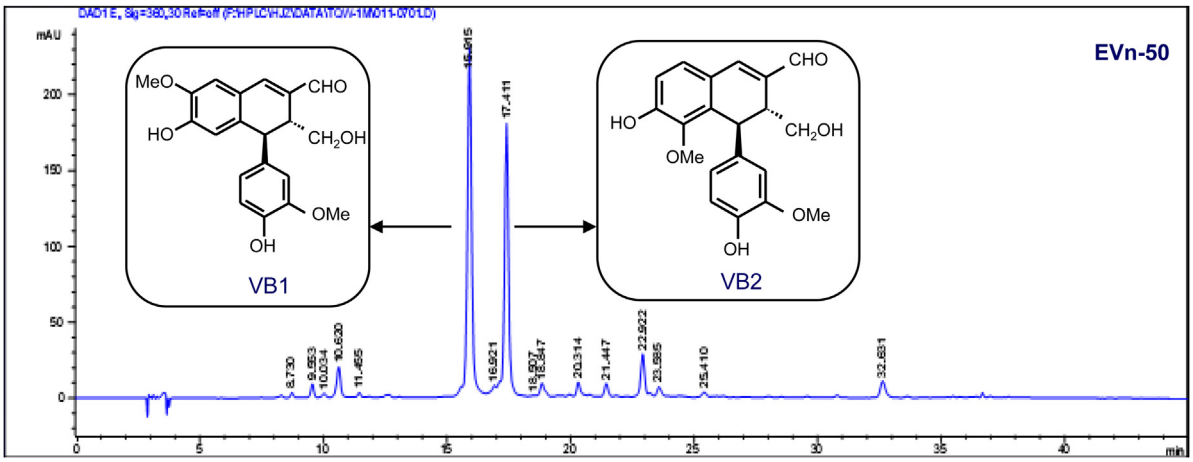

Fig. 1. HPLC analysis of EVn-50 and structures of VB1 and VB2.

EVn-50. Among them, VB1 and VB2 (Fig. 1) are two major lignans and account for 38% and 17% of EVn-50, respectively. As mentioned above, classic plant lignans acquire biological activities *via* their metabolized intermediate mammalian lignans END and ENL (Stattin et al. 2002). Interestingly, Zhou et al. found that lignans mixture EVn-50 failed to generate mammalian lignan after fed to rats, therefore possessing a distinct metabolism properties compared to classic plant lignan (Zhou et al. 2009).

Kuijsten reported that plasma ENLs are associated with lower risk of colorectal cancer (Kuijsten et al. 2006). Recently, there is also a report that Schisandra lignans can prevent hepatic metastasis of mouse mastocytoma P815 tumor cells (Tang et al. 2011). These studies suggest that lignan may be useful for treatment of non-hormone cancers, as well as for hormone related cancers. Hence, this study is aimed to define the spectrum of the cytotoxic activity of lignans mixture EVn-50 and to investigate the potential mechanisms underlying the anticancer action of EVn-50 through assessing the effect of EVn-50 on cancer cell cycle and apoptosis.

Materials and methods

Materials

Rabbit monoclonal antibody against activated caspase-3, PARP (cleaved p85), cyclin B1, cyclin-dependent kinase 1 (Cdk1), phospho-Cdk1 (Tyr15), cell division cycle (Cdc) 25c, and GAPDH were obtained from Santa Cruz (Santa Cruz, CA, USA). The fluorescein isothiocyanate (FITC) labeled antibody for α-tubulin and antibody against phospho-Histone 3 (Ser10) were obtained from Upstate Biotechnology (Lake Placid, NY, USA). Sulforhodamine B (SRB) and 4′,6-diamidino-2-phenylindole (DAPI) was purchased from Sigma (St. Louis, MO, USA); Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay kit was purchased from Beyotime (Suzhou, China).

Cell lines

Breast cancer MDA-MB-435, MCF-7 cells, ovarian cancer SKOV3, HO-8910 cells, pancreatic cancer cell line BXPC-3, campan-1, kidney cancer cell line 786-O, colon cancer cells HCT-116, HT-29, prostate cancer cell lines PC-3, LNCAP, lung cancer A549 cells, and myelogenous leukemia K562 cell line were purchased from ATCC and maintained in Dulbecco's modified Eagle's medium (Invitrogen; Carlsbad, CA, USA) containing 10% fetal calf serum (Invitrogen; Carlsbad, CA, USA). Gastric cancer cells SGC-7901, MKN-45 and liver cancer cell line SMMC-7721, BEL-7402 were obtained from Shanghai Institute of Biochemistry and Cell Biology, and were grown in RPMI 1640 (Invitrogen; Carlsbad, CA, USA) supplemented with 10% fetal calf serum (Invitrogen; Carlsbad, CA, USA). All the cells were routinely cultured in 37 °C and 95% humidity incubator (Thermo Fisher; Pittsburgh, PA) supplement with 5% CO_2.

High performance liquid chromatography (HPLC) analysis of EVn-50 (Fig. 1)

Lignans mixture EVn-50 and its purified lignan compound VB1 extracted from seeds of *Vitex negundo*, a widely used herb in China, were provided by Dr. Yingjun Zhou and prepared as previously report (Zhou et al. 2009). To confirm the lignan components in EVn-50, the sample of EVn-50 was analyzed by an Agilent 1100 HPLC system consisting of a G1312A QuatPump, a G1314A DAD detector, and a G1332A degasser (Agilent, USA). The EVn-50 separation was performed with a Kromasil ODS-1 column (250 mm × 4.6 mm, 5 μm) maintained at 30 °C. The mobile phase was acetonitrile (A) and 0.2% aqueous acetic acid (B) with a gradient program as follows: 0–35 min, linear gradient 15–30% A at a flow rate of 1.0 ml/min. The effluents were monitored by the DAD detector at 360 nm. All sample and standard solutions were filtered through a cellulose acetate membrane filter (0.45 μm, Anpu Co., Shanghai, China) prior to HPLC analysis.

Measurement of cytotoxic activity

The growth inhibitory effect of EVn-50 or VB1 on cells was measured using the SRB assay as Skehan et al. described (Skehan et al. 1990), and the absorbance at 560 nm was detected with a plate reader (SpectraMax, Molecular Devices; CA, USA). The growth inhibition rate was calculated as (1-A560 treated/A560 control) × 100%. The IC50 values were determined by four parameter logit method as described previously (Volund 1978).

Flow cytometric analysis

Cells were treated with various concentrations of EVn-50 or VB1 24 h. After that, cells were collected and washed with phosphate-buffered saline (PBS; pH 7.4), fixed with 70% ethanol in 30% PBS at 4 °C. Then, the cells were incubated with RNaseA solution (20 μg/ml) at 37 °C for 15 min, stained with propidium iodide (Sigma; St. Louis, MO, USA) for 30 min in the dark at room temperature. For each sample at least 1×10^5 cells were analyzed using a FACS-Calibur cytometer (Becton Dickinson; San Jose, CA, USA), and the percentage of cells in each cell cycle phase was calculated using