2,3,6-Tribromo-4,5-dihydroxybenzyl Methyl Ether Induces Growth Inhibition and Apoptosis in MCF-7 Human Breast Cancer Cells

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In this study, we investigated the effects of 2,3,6-tribromo-4,5-dihydroxybenzyl methyl ether (TDB), isolated from Symphyocladia latiscula (marine red algae), on the proliferation of MCF-7 human breast cancer cells. TDB treatment for 48 h inhibited cancer cell growth and induced DNA fragmentation. Furthermore, morphological characterizations such as apoptotic bodies and membrane blebs were shown by electronic microscopy. TDB-induced apoptosis in the MCF-7 cells was closely linked with the down-regulation of Bcl-2 protein expression and the cleavage of caspase-3 substrates, with poly(ADP-ribose) polymerase cleavage occurring by TDB treatment. TDB treatment also caused a marked increase in the level of p21WAF1/Cip1 protein in a p53-dependent manner. In addition, the upregulation of p21WAF1/Cip1 in the MCF-7 cells was related to a decrease in c-Myc protein in a dose-dependent manner. Based on our data, TDB is a good candidate for further evaluation as an effective chemotherapeutic agent, acting through the induction of apoptosis.

Key words: 2,3,6-Tribromo-4,5-dihydroxybenzyl methyl ether, Cytotoxicity, Apoptosis, Human breast cancer cells

INTRODUCTION

Worldwide, breast cancer is the leading cause of death for women between the ages of 40 and 55 years (Baselga et al., 1994). This pathology is currently controlled by surgery and radiotherapy, and is frequently supported by adjuvant chemo- or hormonotherapies (Bange et al., 2001). However, breast cancer is highly resistant to chemotherapy, and there is still no effective cure for patients with advanced stages of the disease, especially in cases of hormone-independent cancer (Bange et al., 2001). An effective chemopreventive treatment would have an important impact on breast cancer morbidity and mortality.

Apoptosis has been characterized as a fundamental cellular activity to maintain the physiological balance of an organism (Hengartner, 2000). It is also involved in the immune defense machinery, and plays a necessary role as a protective mechanism against carcinogenesis, by eliminating damaged cells or excess abnormal cells that have proliferated due to induction by various chemical agents (Fesik, 2005; Li et al., 2005). Emerging evidence has demonstrated that the anticancer activities of certain chemotherapeutic agents are involved in the induction of apoptosis, which is regarded as the preferred way to manage cancer (Fesik, 2005; Hsu et al., 2005).

Many natural products exhibit anti-proliferation, anti-tumor, or anti-inflammation effects. Recent studies have indicated that the marine environment is proving to be a very rich source of bioactive compounds (Mayer and Gustafson, 2003; Newman and Cragg, 2004). Natural marine products contain an abundance of biologically active substances with novel chemical structures and favorable pharmacological activities against human cancers (Choi et al., 2005, 2006; Park et al., 2006, 2007; Zhang et al., 2006). We chose marine red algae as the primary natural marine product for this study, and until now, its biological activities were unknown. 2,3,6-Tribromo-4,5-dihydroxybenzyl methyl ether (TDB) is a natural marine product originally
isolated from *Symphyocladia latiuscula* (Rhodomelaceae). TDB is known to exert various biological activities such as peroxynitrite scavenging and cytoprotective effects (Chung et al., 2001), as well as anti-viral effects (Park et al., 2005). However, the anti-proliferative effect of this compound at non-cytotoxic concentrations on human breast cancer cells has not yet been explored. In our experiments, we demonstrate that TDB markedly inhibits breast cancer cell growth and efficiently induces p53-dependent apoptosis.

### MATERIALS AND METHODS

**Materials**

First, TDB (Fig. 1) was isolated and purified as previously described (Chung et al., 2001; Park et al., 2005); then, it was dissolved in dimethyl sulfoxide (DMSO) and used for subsequent assays, where the final concentration of DMSO was less than 0.01% (v/v), and where there was no influence on the cell growth. Mouse monoclonal antibodies to p53 and poly(ADP-ribose) polymerase (PARP), rabbit polyclonal antibodies to Bax, Bcl-2, and c-Myc, and goat polyclonal antibodies to c-Jun, were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The monoclonal anti-poly(ADP-ribose) polymerase (PARP) antibody was purchased from Calbiochem (Cambridge, MA), and the mouse monoclonal anti-p21WAF1/CIP1 antibody was obtained from Transduction Lab. (Lexington, KY). Peroxidase-labeled donkey anti-rabbit immunoglobulin and peroxidase-labeled sheep anti-mouse immunoglobulin were purchased from Amersham Corp. (Arlington Heights, IL). All other chemicals were of the highest purity as available from Sigma Chemical Co. (St. Louis, MO) and Junsei Chemical Co. (Tokyo, Japan).

**Cell viability assay**

The MCF-7 human breast cancer cells were obtained from the American Type Culture Collection (Rockville, MD). They were routinely cultured in DMEM (Gibco BRL, Grand Island, NY) that was supplemented with 10% fetal bovine serum, 50 mg/ml gentamicin, and 135 mg/ml glutamine. Cell proliferation was assessed using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma] assay, which is based on the conversion of MTT to MTT-formazan by mitochondrial enzymes, as previously described (Tada et al., 1986).

**Assessment of DNA degradation**

The cells were harvested, rinsed twice in cold PBS, and resuspended in lysis buffer [5 mM Tris-HCl (pH 7.5), 5 mM ethylenediaminetetra acetic acid (EDTA), and 0.5% Triton X-100] at 4°C for 30 min. After centrifugation at 27,000×g for 15 min, the supernatant was treated with RNase, followed by proteinase K digestion, and then phenol/chloroform/isooamyl alcohol (25:24:1) extraction and isopropanol precipitation. The DNA was separated through a 1.5% agarose gel, stained with ethidium bromide (EtBr, Sigma), and visualized by an ultraviolet light source.

**Western blot analysis**

The total cell lysates were lyzed in extraction buffer [40 mM Tris (pH 8.0), 120 mM NaCl, 0.5% NP-40, 0.1 mM sodium orthovanadate, 2 mg/mL aprotinin, 2 mg/mL leupeptin, and 100 mg/mL phenylmethylsulfonyl fluoride]. The supernatant was collected and the protein concentration determined with a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Western blot analysis was performed as previously described (Im et al., 2005). The proteins were visualized with an enhanced chemiluminescence (ECL) detection system (Amersham).

**Morphological study of apoptotic cells**

After the cell suspension was treated with or without an IC₅₀ dose of TDB for 48 h, and washed with Hank's solution, it was centrifuged (200 g for 10 min) and fixed as a pellet in 2.5% glutaraldehyde-1% osmium tetroxide buffered with PBS (pH 7.2), and processed for ultrathin sectioning (Choi et al., 2003) by a slightly modified method. The samples were dehydrated in a graded ethanol series, embedded in Spury resin, and morphologically analyzed with standard procedures under a Hitachi H600-3 electron microscope (Tokyo, Japan).

**Statistical analysis**

All data are presented as means ± SD. The mean values were calculated from data obtained by no less than three independent experiments.

### RESULTS

**Effects of TDB on MCF-7 cell growth inhibition**

The ability of TDB to induce cell death was determined in MCF-7 human breast cancer cells. To evaluate TDB's effects on cell viability, the cancer cells were treated with different concentrations of TDB for 48 h, and then MTT assay was performed. Cell viability was significantly reduced with 48 h of TDB treatment in a concentration-dependent manner. The dose required for half-maximal inhibition (IC₅₀) of MCF-7 cell growth was approximately 100 μM (Fig. 2).