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Resveratrol Analog, 3,5,2',4'-Tetramethoxy-*trans*-stilbene, Potentiates the Inhibition of Cell Growth and Induces Apoptosis in Human Cancer Cells

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Resveratrol, a trihydroxystilbene found in grapes and several plants, has been shown to be active in inhibiting multistage carcinogenic process. Using resveratrol as the prototype, we synthesized several analogs and evaluated their growth inhibitory effect using cultured human cancer cells. In the present report we show that one of the resveratrol analogs, 3,5,2',4'-tetramethoxy-*trans*-stilbene, potentiated the inhibition of cancer cell growth. Prompted by the strong growth inhibitory activity of the compound (IC_{50}; 0.8 μg/ml) compared to resveratrol (IC_{50}; 18.7 μg/ml) in cultured human colon cancer cells (Col2), we performed an action mechanism study using the compound. The compound induced the accumulation of cellular DNA contents in the sub-G0 phase DNA contents of the cell cycle by in a time-dependent manner. The morphological changes were also consistent with an apoptotic process. This result indicated that the compound induced apoptosis of cancer cells, and may be a candidate for use in the development of potential cancer chemotherapeutic or cancer chemopreventive agents.

Key words: 3,5,2',4'-Tetramethoxy-*trans*-stilbene, Growth inhibition of cancer cells, Apoptosis, Cancer chemoprevention

INTRODUCTION

Resveratrol (3,5,4'-trihydroxy-*trans*-stilbene), a naturally occurring phytoalexin that is present in grapes, peanuts, pines and other several plants, has been reported to exhibit a variety of important biological effects including a protective role of atherosclerosis and coronary heart diseases (Frenkel *et al.*, 1993; Pace-Asciak *et al.*, 1995). Many studies have also demonstrated the potential of resveratrol to mediate the strong antioxidant, antimutagenic, anti-inflammatory, or potent cancer chemopreventive effects in carcinogenesis (Jang *et al.*, 1997; Uenobe *et al.*, 1997). In addition, resveratrol exhibited growth inhibitory effects on several human cancer cell lines, including human oral squamous carcinoma, promyelocytic leukemia, breast, and prostate cancer cells (Elattar *et al.*, 1999; Surh *et al.*, 1999; Lu *et al.*, 1999; Hsieh *et al.*, 1999). In view of the inhibitory potential of resveratrol in carcinogenesis, we have synthesized additional stilbene analogs in order to develop more potent novel cancer chemopreventive or chemotherapeutic agents. At the present time we report that one of resveratrol analogs, 3,5,2',4'-tetramethoxy-*trans*-stilbene, strongly inhibited the growth of human cancer cells and induced apoptosis compared to resveratrol itself.

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MATERIALS AND METHODS

Chemicals

Trichloroacetic acid (TCA), sulforhodamine B (SRB), propidium iodide, trypsin inhibitor, bisbenzimide (Hoechst 33258), and RNase A were purchased from Sigma Chemical Co. (St. Louis, MO). Minimal essential medium with Earles's salt (MEME), fetal bovine serum (FBS), non-essential amino acid solution (10 mM, 100X), trypsin-EDTA solution (1X) and antibiotic-antimycotic solution (PSF) were purchased from GIBCO-BRL (Grand Island,

NY). 3,5,2',4'-tetramethoxy-*trans*-stilbene (Fig. 1) was synthesized by employing the Witthig reaction between a 2,4-dimethoxybenzaldehyde and a triphenyl(3,5-dimethoxybenzyl)phosphonium bromide as described by Ali *et al.* (Ali *et al.*, 1992), which was provided by Dr. Sanghee Kim (Natural Products Research Institute, Seoul National University, Korea).

Evaluation of growth inhibitory potential with human cancer cell lines

The cancer cell growth inhibitory potential was determined as described previously (Lee *et al.*, 1998). Briefly, human lung carcinoma (A549, which was obtained from ATCC) or human colon carcinoma (Col2, which was obtained from the Department of Surgical Oncology, University of Illinois at Chicago) cells (in the log growth phase) were counted, then diluted to 5×10^4 cells/ml by fresh medium, and added to 96-well microtiter plates (190 μl/well) which contained test materials (10 μl in 10% aqueous DMSO). The test plates were incubated for 3 days at 37°C in a CO_2 incubator. For the zero day controls, cells were incubated for 30 min at 37°C in a CO_2 incubator. All treatments were performed in triplicate. After the incubation periods, the cells were fixed by the addition of 50 μl of a cold 50% aqueous TCA solution (4°C for 30 min), washed 4-5 times with tap water, and then air-dried. The fixed cells were stained with SRB (0.4% w/v in 1% aqueous acetic acid) for 30 min. Free SRB solution was then removed by rinsing with 1% acetic acid. The plates were then air-dried, the bound dye was solubilized with 200 μl of 10 mM tris-base (pH 10.0), and the absorbance was determined at 515 nm using an ELISA plate reader. Finally, the absorbance values that were measured in each of the treatment procedures were averaged, and the average value that was obtained for the zero day control was subtracted. These results were expressed as a percentage, that was relative to the solvent-treated control incubations, and the IC_{50} values were calculated using non-linear regression analyses (percent survival versus concentration).

Nuclear staining assay

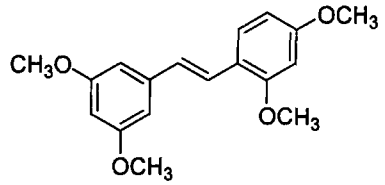
Human colon carcinoma cells (Col2, 2×10^6 cells) were plated on 10 cm culture dishes, and were treated with the test agents for 24 h. Floating cells were collected by centrifugation at 2000 × g for 5 min and attached cells were trypsinized and then harvested by centrifugation. The two cell parts were combined and were washed with cold PBS and then fixed with 90% ethanol. The fixed cells were stained with Hoechst 33258 for 30 min. The cells were then washed with PBS twice and placed on slides. Morphological change was observed under a confocal microscope (Zeiss, LSM 510).

Analysis of cell cycle dynamics by flow cytometry

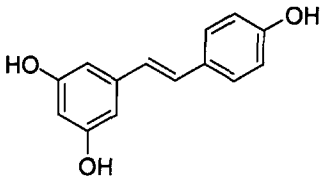
Cell cycle analysis by flow cytometry was performed as previously described (Lee *et al.*, 1998). Briefly, Col2 cells were plated at a density of 2×10^6 cells per 10 cm on culture dishes and incubated for 24 h. Fresh media containing test samples were added to the culture flasks. After a 24 h incubation, the cells were harvested (trypsinization and centrifugation), fixed with 90% ethanol, and incubated with a staining solution containing 0.2% NP40, RNase A (30 μg/ml), and propidium iodide (50 μg/ml), in a phosphate-citrate buffer (pH 7.2). The cellular DNA content was analyzed by flow cytometry using a Becton Dickinson laser-based flow cytometer. At least 30,000 cells were used for each analysis, and the results were displayed as histograms. Percentages of cells in the sub-G0, G0/G1, S, and G2/M phases of the cycle were calculated.

RESULTS AND DISCUSSION

Cancer chemoprevention is defined as inhibiting, delaying, or reversing the carcinogenic process using non-toxic chemicals and is considered to be a promising strategy in controlling cancer progression (Sporn and Newton, 1979; Hong and Sporn, 1997). A variety of chemical compounds have been reported to protect against chemical carcinogenesis and thus are considered to be cancer chemopreventive agents (Kelloff *et al.*, 1992; Wattenberg, 1997). Among these, resveratrol is a promising phytochemical agent that has recently attracted interest because of its cancer chemopreventive activity in multistage carcinogenesis. Resveratrol is a naturally occurring polyphenolic phyto-



3,5,2',4'-Tetramethoxy-*trans*-stilbene (Compound 1)



3,5,4'-Trihydroxy-*trans*-stilbene (Resveratrol)

Fig. 1. Chemical structures of 3,5,2',4'-tetramethoxy-*trans*-stilbene and resveratrol

alexin, which has been demonstrated to have cancer chemopreventive activity in *in vivo* animal experiments (Jang *et al.*, 1997; Carbo *et al.*, 1999). In our continuing efforts of searching for novel cancer chemopreventive agents from natural products or from synthetic compounds we have synthesized and tested the effects of resveratrol and its analogs on the growth of human cancer cells (Nam *et al.*, 2000). One analog 3,5,2',4'-tetramethoxy-*trans*-stilbene (compound **1**) exhibited a remarkable growth inhibitory effect against human lung (A549) and colon cancer cells (Col2) (Fig. 2). Compared to resveratrol, compound **1** was approximately ten to twenty times more active than it in lung (IC_{50}: 0.8 μg/ml for compound **1**; 6.9 μg/ml for resveratrol) and in colon cells (IC_{50}: 0.8 μg/ml for compound **1**; 18.7 μg/ml for resveratrol), respectively. These results coincided with the recent report by Lu *et al.* (Lu *et al.*, 2001), which suggested that polymethoxy-stilbenes potentiated the growth inhibition of cancer cells. Furthermore, in order to explore whether or not the growth inhibitory potential of compound **1** is related to apoptosis, the morphological changes were first examined by utilizing the Col2 cells treated with 25 μM compound **1** for 24 h. As illustrated in Fig. 3, cells that were exposed to compound **1** show the distinct morphological features of apoptosis likewise membrane blebbing formation or cell shrinkage. Nuclear staining with the Hoechst 33258 dye which was observed under a confocal microscope also showed a relatively higher nuclear condensation or a fragmentation of cells that were treated with compound **1** compared to non-treated control cells (Fig. 4). Moreover, in order to determine the characteristics of growth inhibition of compound **1** in greater details, flow cytometric analysis was performed. Col2 cells were treated with 5.4 μM compound **1** (equivalent to 2 × IC_{50}) or 44.4 μM resveratrol (equivalent to half of IC_{50}) for up to 48 h and then subjected to an analysis of cell cycle distribution. As shown in Fig. 5, cells treated with compound **1** showed an increased accumulation of the cells in the sub-G0 phase of cell cycle in a time-dependent manner up to 21% by 48 h (Fig. 5B). Resve-

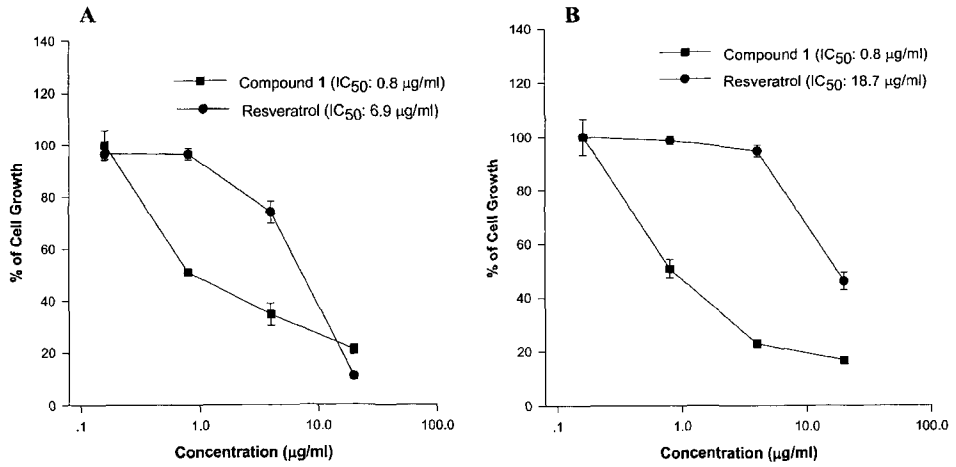


Fig. 2. The growth inhibitory effects of compound **1** and resveratrol on human lung (A549) and colon (Col2) cells. Human lung (A549, A) or colon (Col2, B) cells were plated at 10,000 cells in 96-well plates in MEME supplemented with 10% FBS, and incubated with the test compounds as the indicated concentrations for 3 days as described in the Materials and Methods. The data are represented as the means ± SE (n=3).

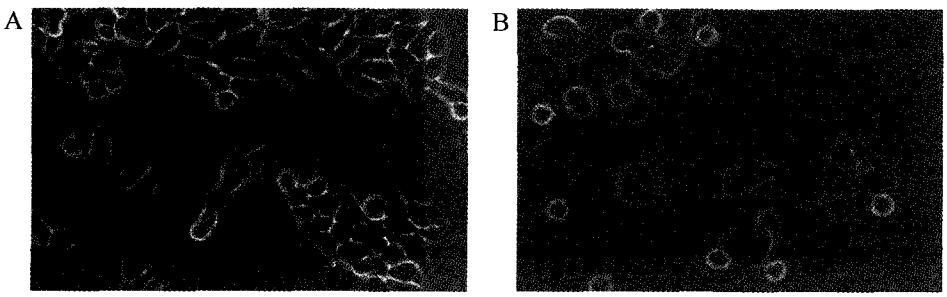


Fig. 3. Morphological change in cultured Col2 cells treated with compound **1**. Morphological changes of Col2 cells treated with DMSO alone (**A**) or with 25 μM compound 1 (B) for 24 h were observed under the phase-contrast microscope and photographed.

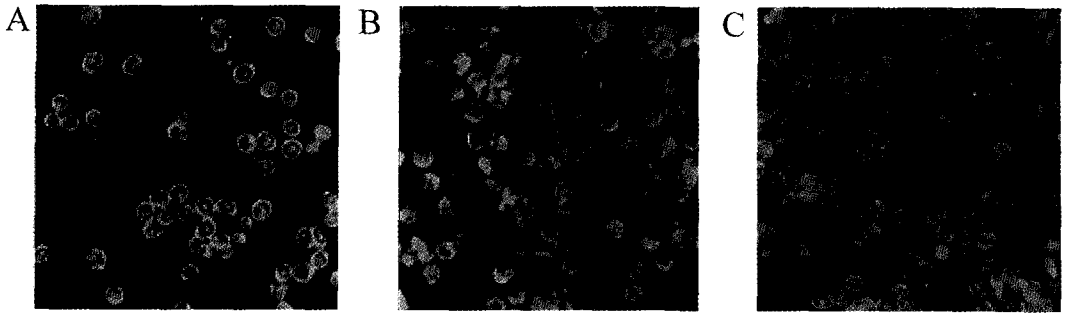


Fig. 4. Nuclear staining examination of Col2 cells treated with compound **1**. Col2 cells were treated with DMSO (A), compound **1** (25 μM, B), or resveratrol (25 μM, C) for 24 h, then stained with the Hoechst 33258 fluorescence dye, and finally observed under a confocal microscope.

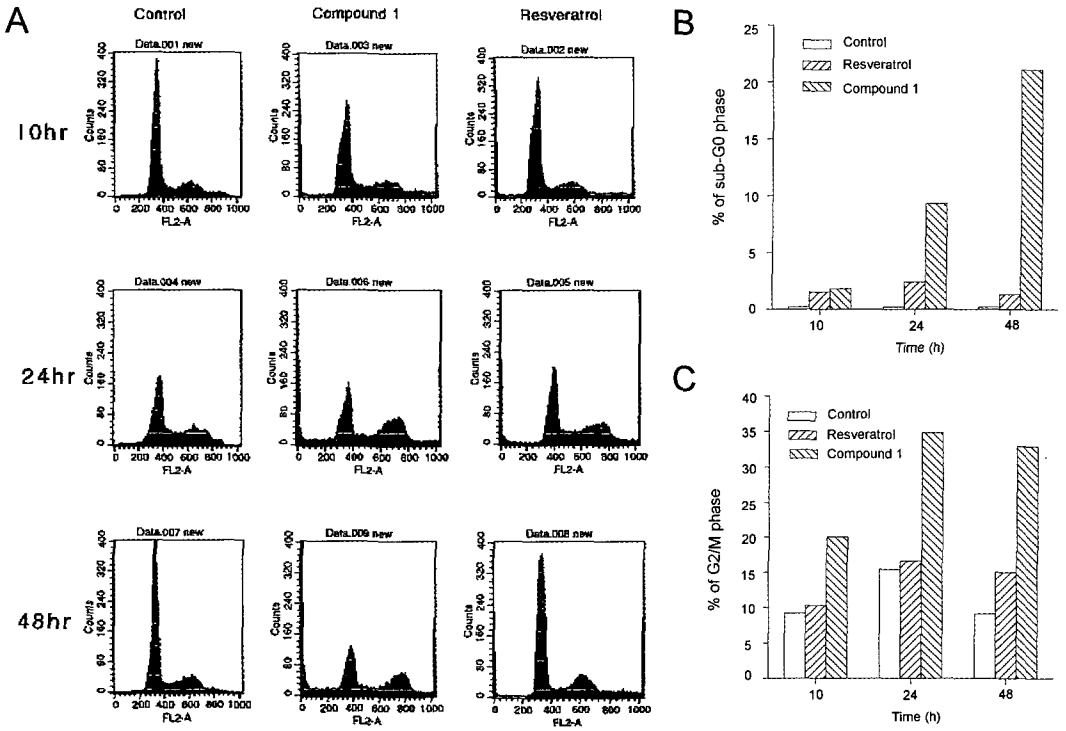


Fig. 5. Effect of compound **1** on cell cycle distribution in cultured Col2 cells. (A) Flow cytometric analysis of the DNA content treated with vehicle-control, resveratrol (44.4 μM), or compound **1** (5.4 μM) for 10, 24, or 48 h. (B) The fraction of cells in the sub-G0 phase of compound **1** at the indicated time. (C) The fraction of cells in the G2/M phase of the control or compound **1** treated cells.

ratrol, however, showed a very low accumulation at the sub-G0 phase, even when the cells were treated with over an 8 times higher concentration compared to compound **1**. A large proportion of the cells accumulated in the G2/M phase of the cell cycle at 24 and 48 h that were treated with compound **1** (Fig. 5C), indicated a possible growth arrest at the M/G1 phase transition period. Therefore, the induction of apoptosis mediated by compound **1** could primarily be correlated with the accumulation of G2/M phase of the cell cycle and the arresting of the cell growth at the M/G1 phase transition. Many studies of the use of resveratrol for cell cycle distribution and apoptosis revealed a dependence on the concentration of the compound, the duration of treatment, or the cell types (Surh *et al.*, 1999; Schneider *et al.*, 2000; Park *et al.*, 2001). In our experiment, however, resveratrol did not show a potential the induction of apoptosis in cultured colon cells.

Overall, the present study suggests that the resveratrol analog 3,5,2',4'-tetramethoxy-*trans*-stilbene (compound **1**) is a more potent inhibitor of the growth of cancer cells compared to resveratrol. The growth inhibition of the compound is highly related to induction of apoptosis by means of cell cycle arrest at the transition of the M/G1

phase. Further studies are needed to evaluate the precise apoptosis-inducing activity of compound **1** in diverse cancer cell lines or biomarker assays related to apoptosis. On the basis of the effect of resveratrol as a cancer chemopreventive agent, the potential activity of the synthetic compound **1** as an inducer of apoptosis should be considered for further investigation for its development as a cancer chemopreventive agent.

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