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Resveratrol derivatives potently induce apoptosis in human promyelocytic leukemia cells

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Abbreviations: CYP, cytochrome P450; HL-60, human promyelocytic leukemia

Abstract

Resveratrol has been shown to possess antioxidant and anticancer activities, but little is known on the effect of resveratrol derivatives. Recently we have isolated resveratrol and its dimers and trimers from peony (*Paeonia lactiflora*) seeds, and reported their strong antioxidant and cytotoxic activity. In the present study, we have evaluated cellular effects of resveratrol derivatives; viniferin, gnetin H, and suffruticosol B on the proliferation and apoptosis in HL-60 cells *in vitro*. All resveratrol and its derivatives reduced viability of HL-60 cells in a dose-dependent manner with their IC_{50} values of 20-90 μM. Ascending orders of IC_{50} values were suffruticosol B, gnetin H, viniferin and resveratrol respectively. HL-60 cells treated with the four stilbenes exhibited the distinct morphological changes characteristics of cell apoptosis such as chromatin condensation, apoptotic bodies, and DNA fragmentations. A time-dependent histogram of the cellular DNA analyzed by flow cytometry revealed a rapid increase in subdiploid cells and a concomitant decrease in diploid cells exposed to 100 μM resveratrol for 0-24 h. Cells treated with 25 μM of resveratrol, viniferin, gnetin H, and suffruticosol B for 24 h resulted in increment of sub-G1 population by 51, 5, 11 and 59%, respectively. Treatment of cells with 0-20 μM resveratrol for 5 h produced a concentration-dependent decrease in cytochrome P450 (CYP) 1B1 mRNA levels. Suffruticosol B also suppressed CYP1B1 gene expression. These results demonstrated that resveratrol oligomers also strongly suppressed HL-60 cell proliferation, and induced DNA damage. In addition, CYP1B1 gene supression may suggest an involvement in the resveratrol-induced apoptosis in HL-60 cells.

Keywords: antioxidants; apoptosis; cell death; neoplasm; paeonia

Introduction

Resveratrol, *trans*-3,5,4'-hydroxystilbene, is a naturally occurring phytoalexin and a polyphenolic compound. Resveratrol has estrogenic activity in mammals (Gehm *et al*., 1997; Bowers *et al*., 2000) and therefore is classified as a phytoestrogen. Resveratrol has recently attracted considerable interest because of its inhibitor activity on multiple cellular events associated with carcinogenesis (Jang *et al*., 1997; Runqing *et al*., 1999). Resveratrol has been shown to have potent growth inhibitory effects on various cancer cells such as colonic tumor cells (Schneider *et al*., 2000), leukemic cells (Gautam *et al*., 2000; Dorrie *et al*., 2001), breast and prostate cancer cells (Mitchell *et al*., 1999; Damianaki *et al*., 2000), and has been suggested as one of the most promising cancer chemopreventive agents.

Resveratrol was found to be present in a number of plant species including grapes and peanuts. Recently we have isolated resveratrol and its dimers and trimers such as viniferin, gnetin H, and suffruticosol A and B from peony (*Paeonia lactiflora*) seeds, known as one of the richest sources of various resveratrol derivatives (Figure 1; Kim *et al*., 2002a). Resveratrol oligomers have thus far been isolated mainly from five plant families, namely *Dipterocarpaceae, Vitaceae, Cyperaceae, Gnetaceae* and *Leguminosae*. Most of resveratrol oligomers in plants were formed from resveratrol or viniferin by dehydrogenation and Diels Alder reaction (Sotheeswaran and Pasupathy 1993). Resveratrol oligomers like the monomer are known to exert strong antioxidant activity (Bernard *et al*., 1997; Wang *et al*., 1999; Kim *et al*., 2002a) and to inhibit growth of several cancer cell lines (Ohyama *et al*.,

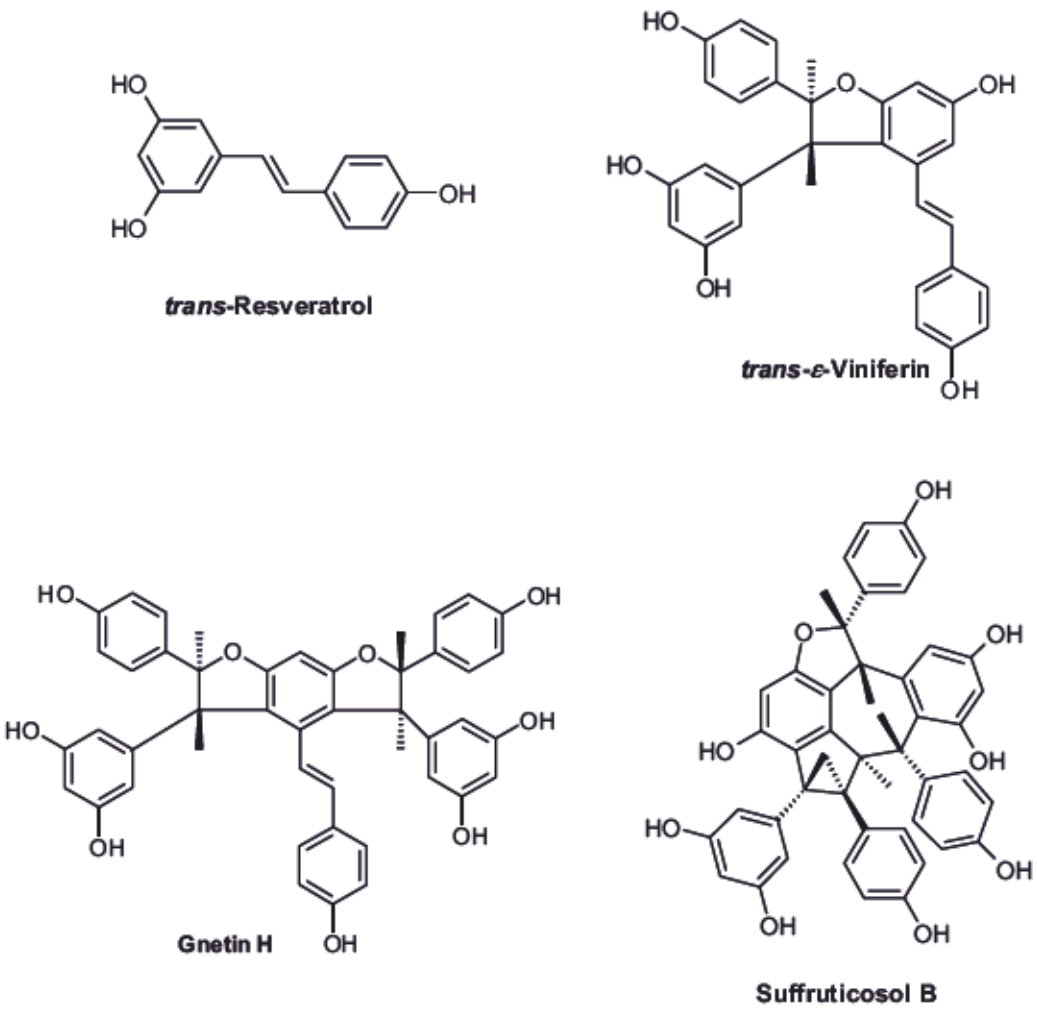

Figure 1. Chemical structure of resveratrol and its oligomers.

1999; Kim *et al.*, 2002b). However, very little is known on the mechanism of antiproliferative effects of resveratrol derivatives on cancer cells.

Recent studies were focused on the apoptosis of cell death, as a probable target mechanism of resevatrol on cancer cells. Apoptosis is a genetically controlled response of an intrinsic cellular system that is required for a critical balance between cell proliferation and cell death in normal development and maintenance of homeostasis of organisms (Krammer *et al.*, 1994). Many chemotherapeutic agents suppress growth of transformed or malignant cells by inducing apoptosis (Thompson *et al.*, 1995; Hannun, 1997; Park *et al.*, 1997). An induction of tumor cell apoptosis is one of the efficient target for drug development (Shi *et al.*, 1996; Watabe *et al.*, 1996) and has become a major focus in the study of cancer therapy (White, 1996). The present study showed that resveratrol dimers and trimers isolated from peony seeds inhibit the growth and induce apoptosis in human leukemia HL-60 cells.

Materials and Methods

Chemicals

Propidium iodide (PI), 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) and RNAse A were purchased from Sigma Chemical Co. (St. Louis, MO). FBS was from Hyclone (Logan, UT). Antibiotics-antimycotes, RPMI 1640 and trypsin-EDTA were from Gibco BRL (Gland Island, NY). Hoechst 33342 was from Calbiochem (San Diego, CA). Resveratrol and its derivatives were isolated from seeds of *Paeonia lactiflora* Pall and their chemical structures were confirmed by mass and nuclear magnetic resonance spectroscopy as described previously (Kim *et al.*, 2002a). Stock solution of resveratrol and its derivatives were made in DMSO at a concentration of

10 mmol/l.

Cell culture and cytotoxicity assay

Human promyelocytic leukemia (HL-60) cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 in RPMI 1640 supplemented with 10% heat inactivated FBS, 20 mM Hepes (pH 7.0) and 1% antibiotic-antimycotic. The cytotoxic effect of resveratrol and its derivatives was analyzed by MTT assay, which was known as a method to determine the viability of cells by staining viable cells with MTT. For MTT assay, HL-60 (5×10^4 cell/well) were grown for 2 days with serial dilutions of resveratrol derivatives in 96 well plate. After incubation, 50 µl of MTT solution (1.1 mg/ml) was added to each well and then incubated for an additional 4 h. After centrifugation, the supernatant was removed from each well. The colored formazan crystal produced from MTT was dissolved in 150 µl of DMSO and then the optical density value was measured at 540 nm by a plate reader (Multiscan MCC/340, Labsystem, Finland).

Morphological examination

HL-60 cells (1×10^6 cells/ml) grown in a 6-well plates were treated with 100 µM of four stilbenes at 37°C for 24 h. Morphological changes occurring in the cells were observed under phase-contrast microscope and photographed. Ultrastrutual examination was performed by conventional techniques using a biological inverted microscope (Nikon T-300, Tokyo, Japan). For nuclear staining, HL-60 cells (1×10^6 cells/ml) were cultured in a 6-well plates in RPMI 1640 medium containing 10% FBS in the absence or presence of 25 µM resveratrol and its derivatives. After 24 h, cells were stained with the DNA specific fluorochrom Hoechst 33342 (10 µM) for 30 min, and observed under fluorescent microscopy (Axioplan2 imaging and Aiophot2 Universal Microscope, Zeiss, Hallbergmoos, Germany).

Analysis of DNA fragmentation

HL-60 cells at a density of 1×10^6 cells/ml were treated with 25 µM resveratrol and its derivatives for 24 h, respectively. The isolation of apoptotic DNA fragments was performed as described by Herrmann *et al.* (1994). Briefly, the cells were harvested by centrifugation at 1,200 rpm for 3 min and then treated with a lysis buffer (1% Nonidet P-40, 20 mM EDTA, 50 mM Tris-HCl, pH 7.5). After centrifugation for 5 min the supernatant was collected and brought to 1% SDS and treated for 2 h at 50°C with RNase A and subsequently with proteinase K for 2 h at 37°C. The DNA fragments were precipitated with 2.5 volumes of ethanol in the presence of 5 M ammonium acetate. The DNA fragmentation was visualized by electrophoresis on a 1.6% agarose gel.

Flow cytometry analysis

HL-60 cells were either untreated or treated with 25 µM or 100 µM resveratrol and its derivatives for 24 h. After these treatments, approximately 1×10^6 cell pellets were prepared and washed twice with PBS without Ca^{2+} and Mg^{2+} plus 2% FBS, suspended in 100 µl of PBS, and then 200 µl of 95% cold ethanol was added while vortexing. The cells were incubated at 4°C for 1 h, washed twice with PBS containing 2% FBS and resuspended with 12.5 µg of RNase in 250 µl of 1.12% sodium citrate buffer (pH 8.45). Incubation was continued at 37°C for 30 min before staining of the cellular DNA with 250 µl of propidium iodide (50 µg/ml) for 30 min at 4°C. The stained cells were analyzed on a fluorecent activated cell sorter (FACScan) flow cytometer (Becton & Dickinson, San Jose, CA) for the relative DNA content, based on an increased red fluorescence.

Isolation of tatal RNA and reverse transcription-PCR

Total RNA was isolated from HL-60 cells using the Trizol™ reagent according to the manufacturer's protocol. Ten µg of RNA was reverse transcribed by oligo (dT)15 primer and M-MLV reverse transcriptase for 90 min at 37°C. Sequences for the forward (5'-CAC-TGC-CAA-CAC-CTC-TGT-CTT-3') and reverse (5'-CAA-GGA-GCT-CCA-TGG-ACT-CT-3') PCR primer for cytochrome P450 (CYP)1B1 (Tomas *et al.*, 2000) and those for the forward (5'-TGA-GAA-CGG-GAA-GCT-TGT-CA-3') and reverse (5'-GGA-AGG-CCA-TGC-CAG-TGA-3') primers for GAPDH were used. PCR amplification for CYP1B1 was performed 25 cycles (denaturation at 95°C for 30 s, annealing at 59°C for 30 s, polymerization at 72°C for 45 s). The reactions for GAPDH were performed 25 cycles (denaturation at 95°C for 1 min, annealing at 56°C for 1 min, polymerization at 74°C for 90 s). The amplified cDNA by PCR was visualized by electrophoresis in 1% agarose gel in the presence of ethidium bromide. The band was quantified using the NIH Image analysis program (NIH, Betheda, MD). The scale of each band was expressed as a ratio of the optical intensity of the CYP1B1 band to that of the GAPDH band.

Results

Cytotoxic effect of resveratrol and its derivatives on HL-60 cells

The effects of resveratrol and its derivatives on the

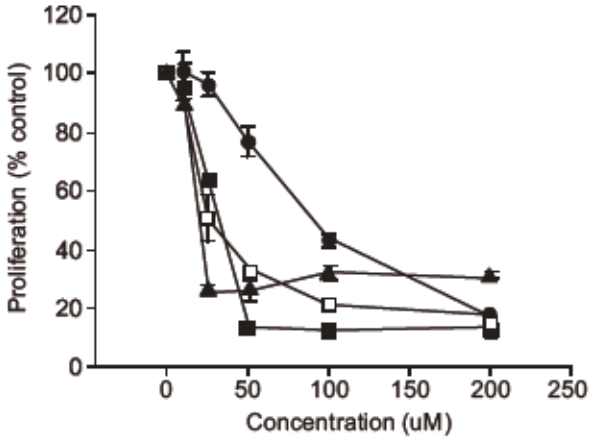

Figure 2. Cytotoxic effect of resveratrol (●), viniferin (□), gnetin H (▲) and suffruticosol B (■) on the growth of HL-60 cells. Cells were grown for 24 h in the presence or absence of different amounts of four stilbenes (10, 25, 50, 100, 200 μM). Error bars represent standard error of assays from 6 replicate experiments.

viability of HL-60 cells determined by MTT assay are shown in Figure 2. When cells were incubated with several doses of resveratrol and its derivatives, ranging from 10 to 200 μM for 24 h, cell viability was decreased in a dose-dependent manner. The concentrations of resveratrol and its derivatives needed to inhibit cell growth of HL-60 cells were quite low. Their IC_{50} values in ascending order were suffruticosol B, gnetin H, viniferin and resveratrol (20, 25, 33, and 95 μM, respectively).

Apoptosis induced by *trans*-resveratrol and its derivatives

HL-60 cells treated with 100 μM resveratrol and its derivatives for 24 h, stained with a DNA binding dye Hoechst 33342 and morphological changes were examined with fluorescence microscope, exhibited typical morphological features of apoptosis such as chromatin condensation, the nuclei fragmentation into various sizes and collapse of the cell into apoptotic body (Figure 3A). DNA fragmentation was evident in most cells as seen by the induction of DNA strand-breakage (Figure 3B). Agarose gel electrophoresis of DNA from the HL-60 cells incubated with resveratrol and its oligomers showed internucleosomal DNA degradation into oligonucleosomal sizes, ranging from 185 bp and its multiples up to 2,500 bp.

Flow cytometric analysis of DNA content again corroborated the apoptotic state of the treated cells. Time course histograms of DNA content in cells treated with 100 μM resveratrol for various periods of time showed a rapid increase in the sub-G1 peak of cells and a concomitant decrease in diploid cells as

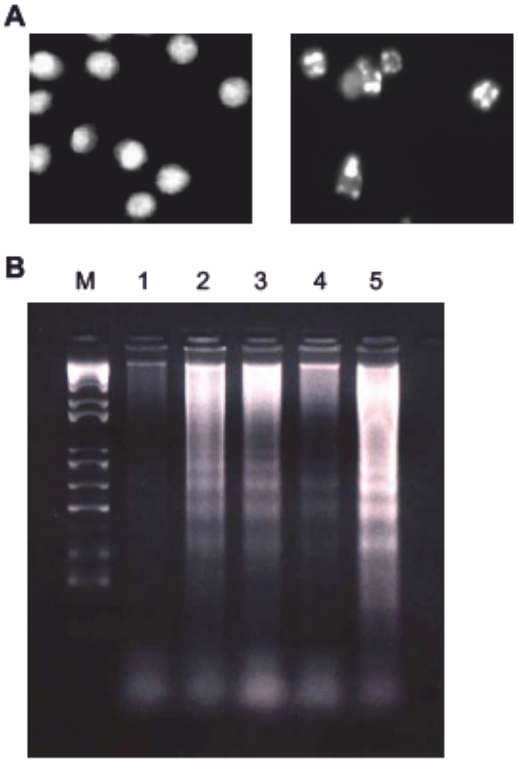

Figure 3. Resveratrol derivatives-induced apoptosis in HL-60 cells. (A) Representative nuclear morphology of HL-60 cells grown for 24 h in the absence (left) or presence (right) of 25 μM resveratrol or its derivatives, and stained with bisbenzimide (Hoechst 33324). Nuclei of cells untreated or treated with resveratrol oligomers were assessed in an Axioplan2 imaging fluorescence. (B) Nucleosomal DNA fragmentation of HL-60 cells after 24 h exposure to 25 μM resveratrol (lane 2), viniferin (lane 3), gnetin H (lane 4) and suffruticosol B (lane 5). Lane M represents 1-kb ladder used as a molecular marker. Lane 1. The DNA fragmentation was analyzed by NP-40 lysis method using 1.6% agarose gel electrophoresis and staining with ethidium bromide.

μM resveratrol, viniferin, gnetin H, and suffruticosol B for 24 h, the percentage of cell populations with subdiploid DNA content increased to 51, 5, 11 and 59, respectively (Figure 4). With a higher concentration of 100 μM, the subdiploid cells increased to >60% of cells treated with resveratrol, viniferin and suffruticosol B, but was about 35% of cells treated with gentin H.

Effects of resveratrols on CYP1B1 mRNA expression

The possible influence of resveratrol and its oligomers on the constitutive CYP1B1 gene expression was investigated. RT-PCR analysis of the concentration-response experiment showed that CYP1B1 mRNA level decreased approximately 30% at higher doses of 10-20 μM resveratrol (Figure 5A). When 20 M of

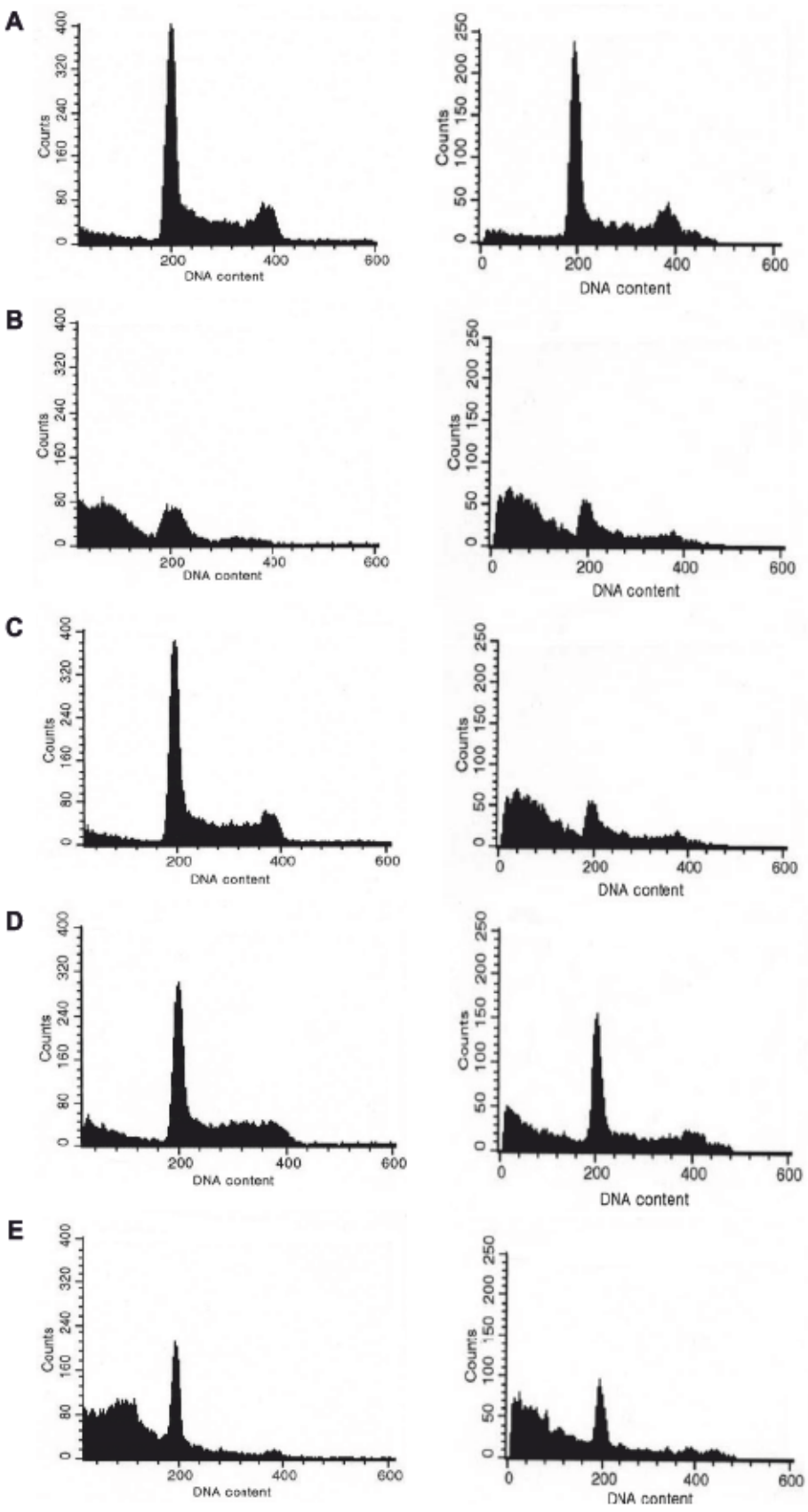

Figure 4. Flow cytometric analysis of cell cycle distribution in HL-60 cells treated with 25 μM (left) and 100 μM (right) resveratrol and its derivatives (A: control, B: resveratrol, C: viniferin, D: gnetin H, E: suffruticosol B) for 24 h. Cell cycle analysis was performed on an equal number of cells (10^6) were fixed and staining of DNA by propidium iodide.

Table 1. Flow cytometric analysis of cell cycle distribution in HL-60 cells treated with 100 μM resveratrol for the time indicated.

Time (h)	Cell cycle analysis (%)			
	Sub G1	G1	S	G2/M
0	9.0	50.3	24.5	15.0
6	40.9	42.1	9.4	6.7
16	49.4	36.9	6.8	6.3
24	70.5	21.3	4.0	3.7

the four stilbenes was treated for 5 h, resveratrol and suffruticosol B, but not viniferin and gnetin H, markedly suppressed CYP1B1 mRNA expression (Figure 5B).

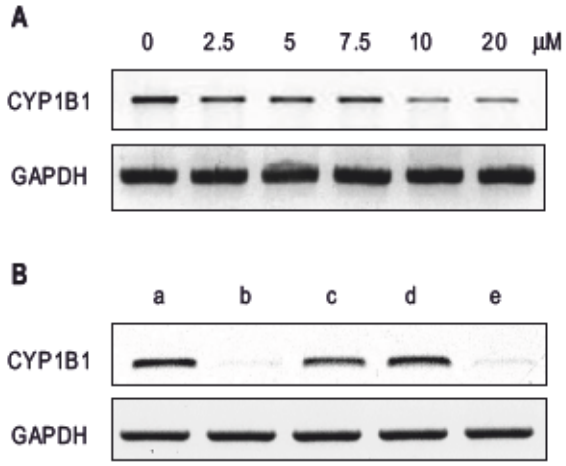

Figure 5. Cytochrom P450 1B1 (CYP1B1) gene expression in HL-60 cells treated with 2.5-20 μM resveratrol (A) and vehicle (a), 20 μM resveratrol (b), viniferin (c) and suffruticosol B (d) for 5 h (B). RT-PCR was carried out with 10 μg of RNA as described in Materials and Methods.

Discussion

The present study demonstrated that like resveratrol, its dimers and trimers such as viniferin, gnetin H, and suffruticosol B also exerted strong cytotoxic activity in human leukemia HL-60 cells and induced apoptosis. We recently observed that resveratrol oligomers isolated from peony seeds inhibited growth of C6, HepG2, Hela, MCF-7 and HT-29 cancer cells in a concentration-dependent manner (Kim *et al*., 2002b). Ohyama *et al*. (1999) also evaluated several resveratrol oligomers isolated from plants, and found hopeaphenol, a resveratrol trimer, showed potent cytotoxicity against a few human cancer cell lines. Cytotoxic activities of resveratrol oligomers were variable in different cancer cell types.

In the present study, HL-60 cell death induced by resveratrol and its derivatives was due to the apoptosis. After treatment of resveratrol and its derivatives, we observed the typical morphological characteristics of apoptosis, such as chromatin condensation and widespread formation of apoptotic bodies. In addition, internucleosomal DNA fragmentation as determined by agarose gel electrophoresis and kinetic analysis of cell cycle distribution by flow cytometry also clearly revealed that resveratrol and its derivatives rapidly induced apoptotic cell death. Previous studies showed the apoptotic effects of resveratrol in HL-60 cells (Surh *et al*., 1999; Joe *et al*., 2002) and in melanoma cells (Niles *et al*., 2003), and vaticanol C, a resveratrol tetramer, in colon cancer cell lines (Ito *et al*., 2002). Howerver, the present study is the first demonstration of resveratrol dimers and trimers-induced potent apoptotic HL-60 cell death.

Among four stilbenes examined, results of the MTT assay showed that suffruticosol B, a resveratrol trimer, exhibited the most potent inhibitory activity with IC_{50} value of 20 μM, whereas IC_{50} value of resveratrol was approximately 95 μM. However, the FACS analysis showed that treatment of 25 μM resveratrol and suffruticosol B for 24 h induced similar degree of extensive apoptosis. Reasons for the different sensitivity of resveratrol on inhibiting cell growth and inducing apoptosis in HL-60 cells are not clear, but might be due at least partly to methodological pitfalls in the use of MTT assay for evaluating cell growth. It has been recognized that phytoestrogens such as resveratrol (Bernhard *et al*., 2003) and genistein (Pagliacci *et al*., 1993) enhance mitochondrial MTT-reducing activity at low concentration ranges of 5-20 μM without a corresponding increase in the number of living cells. This phenomenon may underestimate growth inhibitory effects of resveratrol in certain cancer cells. However, low concentrations of resveratrol dimer and trimers, not like resveratrol, strongly decreased the MTT-reducing activity in the present study.

Various anticancer agents induce cell cycle arrest in some human cancer cell lines (Shi *et al*., 1996; Ragione *et al*., 1998; Joe *et al*., 2002), suggesting a possible role for cell cycle arrest in apoptosis. Resveratrol has been reported to block the S-G_2 transition and arrest the S phase of the cell cycle in HL-60 cells (Ragione *et al*., 1998; Joe *et al*., 2002). The present study showed a gradual reduction in the proportion of cell cycle in the G_1, S and G_2/M phases with concomitant increase in the percentage of sub-G_1 phase as reported by Surh *et al*. (1999). Since concentrations required for resveratrol to induce apoptosis were often higher than those that induced

cell cycle arrest, we might not observe the arrest in the S-phase prior to apoptosis.

The cytochrome P450 enzyme CYP1B1 has been recognized to be highly expressed in a wide variety of human tumors of different organs, but not detected in the corresponding normal tissues (Murray *et al*., 1997). The CYP1B1 activates procarcinogens into carcinogens (Shimada *et al*. 1996; Kim *et al*., 1998), thus it has been suggested as the cause of tumors and a potential target for chemoprevention strategies. Resveratrol inhibited the catalytic activity and constitutive gene expression of human CYP1B1 in MCF-7 human breast cancer cells (Chang *et al*., 2000). The present study also showed resveratrol-induced suppression of CYP1B1 mRNA expression in a dose-dependent manner in HL-60 cells. When four stilbenes were treated, resveratrol and suffruticosol B, but not viniferin and gnetin H, markedly decreased CYP1B1 mRNA expression. Flow cytometric analysis also showed that resveratrol and suffruticosol B induced a stronger apoptotic cell death than viniferin and gnetin H in HL-60 cells. These results suggest a possibility that resveratrol and its derivatives, particularly suffruticosol B, may protect against toxicity and carcinogenicity induced by compounds that undergo CYP1B1-catalyzed bioactivation. In contrast, Potter *et al*. (2002) recently demonstrated that CYP1B1 catalyzes resveratrol to piceatannol that has antileukemic activity. They hypothesized that the functional role of CYP1B1 is a tumor suppressor enzyme, or 'rescue enzyme' that is a molecular mechanism for the anticancer properties of resveratrol. However, further studies are required to elucidate the functional role of CYP1B1 in cancer cells.

In summary, resveratrol derivatives, particularly suffruticosol B, as shown by resveratrol exerted potent cytotoxic and proapoptotic activities, and suppressed gene expression of CYP1B1 in HL-60 cells. Further studies are required to investigate underlying mechanisms of the apoptosis-inducing activity of resveratrol derivatives in diverse cancer cell lines and to ascertain if resveratrol derivatives exert anticarcinogenic activity *in vivo*.

Acknowledgement

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