Resveratrol Induces Growth Inhibition, S-phase Arrest, Apoptosis, and Changes in Biomarker Expression in Several Human Cancer Cell Lines[1]

Andrew K. Joe, Hui Liu, Masumi Suzui, Muhammet E. Vural, Danhua Xiao, and I. Bernard Weinstein[2]

Herbert Irving Comprehensive Cancer Center [A. K. J., H. L., M. S., M. E. V., D. X., I. B. W.] and Department of Medicine [A. K. J., I. B. W.], College of Physicians and Surgeons of Columbia University, New York, New York 10032

ABSTRACT

Purpose: **We examined the effects of the phytochemical resveratrol in six human cancer cell lines (MCF7, SW480, HCE7, Seg-1, Bic-1, and HL60).**

Experimental Design and Results: **Resveratrol induced marked growth inhibition in five of these cell lines, with IC_{50} values of approximately 70–150 μM. However, only partial growth inhibition was seen in Bic-1 cells. After treatment with 300 μM resveratrol for 24 h, most of the cell lines were arrested in the S phase of the cell cycle. In addition, induction of apoptosis was demonstrated by the appearance of a sub-G_1 peak and confirmed using an annexin V-based assay. Cyclin B1 expression levels were decreased in all cell lines after 48 h of treatment. In SW480 cells, cyclin A, cyclin B1, and β-catenin expression levels were decreased within 24 h. There was a decrease in cyclin D1 expression after only 2 h of treatment, and this persisted for up to 48 h. This decrease was partially blocked by concurrent treatment with the proteasome inhibitor calpain inhibitor I. Using a luciferase-based reporter assay, resveratrol did not inhibit cyclin D1 promoter activity in SW480 cells. Furthermore, using a reverse transcription-PCR-based assay, only a higher dose of resveratrol (300 μM) appeared to decrease cyclin D1 mRNA. Seg-1 cells expressed basal levels of cyclooxygenase-2 (cox-2), which was further induced by resveratrol. Neither basal levels nor induction of cox-2 was detectable in the remaining cell lines. Thus, cox-2 does not appear to be a critical target of this compound.**

Conclusions: **These studies provide support for the use of resveratrol in chemoprevention and cancer therapy trials. Cyclin D1, cyclin B1, β-catenin, and apoptotic index could be useful biomarkers to evaluate treatment efficacy.**

INTRODUCTION

The polyphenolic compound resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) is a naturally occurring phytochemical and can be found in approximately 72 plant species, including food products like grapes, peanuts, and various herbs (1). Its exact physiological function is not known, but it may have roles in protecting plants against fungal infections and in conferring disease resistance. Red wine (1.5–3 mg/liter) and grapes (50–100 μg/g grape skins) are probably its main sources in Western diets. One of its richest sources is the herb *Polygonum cuspidatum*, which has been used in Asian folk medicine. Previous investigations have demonstrated its antioxidant and anti-inflammatory activities, its ability to induce phase II drug-metabolizing enzymes, and its ability to inhibit cyclooxygenase activity and transcription; thus, it has activity in regulating multiple cellular events associated with carcinogenesis (for review, see Ref. 1). It may also have *in vivo* activity in modulating indices of platelet activity and lipid metabolism, which could explain the epidemiological evidence that red wine may decrease coronary heart disease mortality (for a review of its potential benefits in atherosclerotic heart disease, please refer to Ref. 2).

Resveratrol has been shown to have growth-inhibitory activity in several human cancer cell lines and in animal models of carcinogenesis. In HL60 promyelocytic leukemia cells, treatment with resveratrol led to growth inhibition, induction of apoptosis, S-G_2-phase cell cycle arrest, and myelomonocytic differentiation (3, 4). Resveratrol also displayed antiproliferative activity in JB6 mouse epidermal, CaCo-2 colorectal, and A431 epidermoid carcinoma cell lines (5–7). Its effects in breast cancer cell lines are more complicated. Whereas some investigators have demonstrated antiproliferative effects in the MCF7, MDA-MB-231, KPL-1, MKL-F, and T47D cell lines (3, 8, 9), others have demonstrated growth enhancement in T47D and MCF7 cells (10, 11). The latter effect appears to be due to the potential estrogenic effects of resveratrol (10–12). Resveratrol inhibited tumor formation in several animal models of carcinogenesis, including mouse 7,12-dimethylbenz(*a*)anthracene/12-*O*-tetradecanoylphorbol-13-acetate-induced skin cancers (1), azoxymethane-induced colon cancers (13), and transplanted Yoshida rat ascites hepatomas (14). In the mouse skin carcinogenesis model, resveratrol inhibited the three major steps of carcinogenesis, initiation, promotion, and progression (1). However, the precise mechanisms by which resveratrol exerts these antitumor effects are not known.

Limited epidemiological and clinical evidence suggest that resveratrol is well tolerated during human consumption and that it may offer benefits with respect to atherosclerotic heart dis-

Received 8/3/01; revised 12/19/01; accepted 12/21/01.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] Supported by an award (to A. K. J.) from PureWorld Botanicals and an American Association for Cancer Research-Cancer Research Foundation of America Fellowship in Prevention Research award (to A. K. J.).
[2] To whom requests for reprints should be addressed, at Herbert Irving Comprehensive Cancer Center, 701 West 168th Street, HHSC-1509, New York, NY 10032. Phone: (212) 305-6921; Fax: (212) 305-6889; E-mail: weinstein@cuccfa.ccc.columbia.edu.

ease. In a small study of 24 healthy male volunteers, trial participants tolerated the consumption of resveratrol-enriched beverages, but the effects of this compound on lipid metabolism and platelet activity were unimpressive (15, 16). Although resveratrol is available commercially as a dietary supplement, there are no published controlled clinical studies demonstrating either its efficacy or safety in the treatment or prevention of cancer or coronary artery disease.

In the present study, we used a spectrum of six human cancer cell lines to further examine the range of antitumor activity of resveratrol. To obtain insights into its mechanism of action, we examined the effects of resveratrol on cell proliferation, cell cycle distribution, apoptosis, and on the levels of expression of several cell cycle control proteins. Our results provide support for the use of resveratrol in clinical chemoprevention and chemotherapy trials. In addition, we have identified potential surrogate biomarkers, which may serve as intermediate clinical end points in these trials.

MATERIALS AND METHODS

Compounds and Antibodies. Resveratrol was generously supplied by PureWorld Botanicals (South Hackensack, NJ) and isolated from the Chinese herb huzhang (*P. cuspidatum*). The compound was supplied in powder form (*trans* isomer), dissolved (stock solution, 100 mM) in DMSO (Sigma Chemical Co., St. Louis, MO), and added directly to cell culture medium at a final concentration of 0.1–0.3% DMSO. Primary antibodies were obtained from the following companies: (*a*) cyclins A, B1, and D1, Upstate Biotechnology (Lake Placid, NY); (*b*) β-catenin, Transduction Laboratories (Lexington, KY); (*c*) cox-2, Oxford Biomed (Oxford, MI); and (*d*) actin, Sigma Chemical Co. LLnL[3] and PI were obtained from Sigma Chemical Co.

Cell Lines and Cell Culture. Seg-1 and Bic-1, esophageal adenocarcinoma cell lines established from patients with Barrett's esophagus, were developed and generously provided by Dr. David G. Beer (University of Michigan, Ann Arbor, MI). Human SW480 colon carcinoma and MCF7 breast carcinoma cell lines were obtained from the American Type Culture Collection (Manassas, VA). The Seg-1, Bic-1, SW480, and MCF7 cells were grown in DMEM (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum (Life Technologies, Inc.). The HCE7 human esophageal squamous carcinoma (17, 18) and HL60 promyelocytic leukemia cells were grown in RPMI 1640 (Life Technologies, Inc.) with 10% fetal bovine serum. All of the cell lines were maintained at 37°C in a 5% CO_2 atmosphere.

Cell Proliferation Assays. Cell proliferation was measured using the MTT Cell Proliferation Kit I (Boehringer Mannheim, Indianapolis, IN), which colorimetrically measures a purple formazan compound produced by viable cells. Cells were plated in flat-bottomed, 96-well microtiter plates (4×10^3 cells/6.4-mm-diameter well). After 12–24 h, cells were treated with DMSO (0.1–0.3%) or increasing doses of resveratrol. After 48 h of treatment, cells were treated with 10 μl of MTT reagent for 4 h at 37°C and then treated with 100 μl of solubilization solution at 37°C overnight. The quantity of formazan product was measured using a spectrophotometric microtiter plate reader (Dynatech Laboratories, Alexandria, VA) at 570 nm wavelength. Results were expressed as a percentage of growth, with 100% representing control cells treated with DMSO alone. All experiments were performed in duplicate.

Apoptosis Assays. The percentage of cells actively undergoing apoptosis was determined using annexin V-PE-based immunofluorescence, as described previously (19). Briefly, cells were plated in 10-cm culture dishes at concentrations determined to yield 60–70% confluence within 24 h. Cells were then treated with either DMSO (0.1–0.3%) or resveratrol (300 μM). After 48 h of treatment, both adherent and floating cells were harvested and then double-labeled with annexin V-PE and 7-aminoactinomycin (PharMingen, San Diego, CA), as described by the manufacturer. Cells were analyzed using a FACScan instrument equipped with FACStation running Cell Quest software (Becton Dickinson, San Jose, CA). All experiments were performed in duplicate and yielded similar results.

Cell Cycle Distribution Analysis. PI staining was used to analyze DNA content. Cells were plated in 10-cm culture dishes at concentrations determined to yield 60–70% confluence within 24 h. Cells were then treated with either DMSO (0.1–0.3%) or resveratrol (300 μM). After a 24-h treatment, both adherent and floating cells were harvested, and the cells were labeled with PI using previously described methods (20). Briefly, cells were resuspended in PBS, fixed with 70% ethanol, labeled with PI (0.05 mg/ml), incubated at room temperature in the dark for 30 min, and filtered through 41-μm spectra/mesh nylon filters (Spectrum, Rancho Dominguez, CA). DNA content was then analyzed using a FACScan instrument equipped with FACStation running Cell Quest software (Becton Dickinson). All experiments were performed in duplicate and yielded similar results.

Protein Extraction and Western Blotting. The methods for protein extraction and Western blot analysis have been described previously (21). Briefly, cells were treated with 0.1–0.3% DMSO (negative control) or resveratrol (300 μM). Experiments with SW480 cells also included coculture with LLnL (100 μM), as described in the Fig. 8 legend. After 2–48 h of treatment, cell lysates were prepared, and 30–60 μg of protein were separated by SDS-PAGE (10%). After transfer to nitrocellulose membranes (Millipore, Bedford, MA), blots were blocked with 5% milk protein, incubated for 1 h with the indicated primary antibody, and then reincubated for 1 h with the corresponding horseradish peroxidase-conjugated secondary antibody. Protein-antibody complexes were detected by the enhanced chemiluminescence system (Amersham, Piscataway, NJ). Immunoblotting for actin was performed to verify equivalent amounts of loaded protein.

Luciferase-based Cyclin D1 Promoter Activity Assays. SW480 cells were seeded in triplicate in 6-well (3.5-cm-diameter) cell culture plates (Becton Dickinson) at a concentration of 1×10^5 cells/well. After 24 h, cells were transfected using

[3] The abbreviations used are: LLnL, calpain inhibitor I; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; cox-2, cyclooxygenase-2; PI, propidium iodide; PE, phycoerythrin; RT-PCR, reverse transcription-PCR; ER, estrogen receptor; cdk, cyclin-dependent kinase.

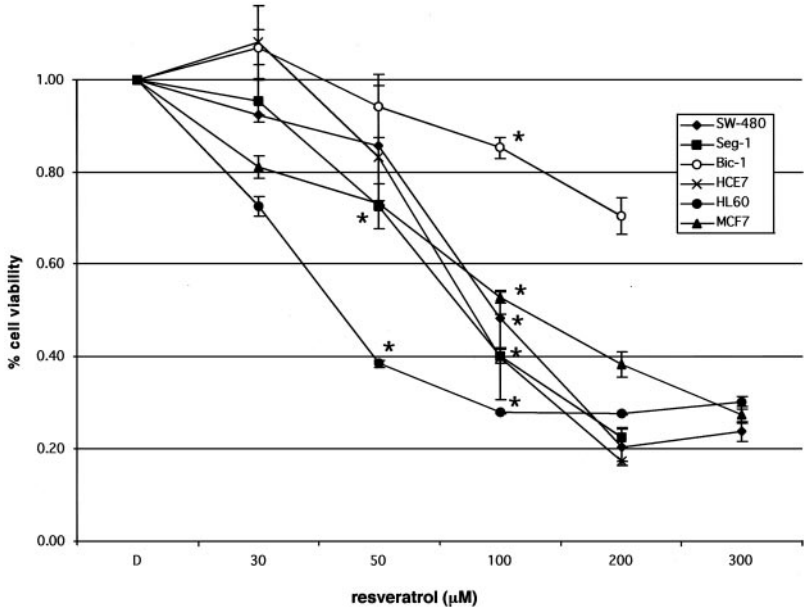

Fig. 1 Growth inhibition of various human carcinoma cells after treatment with resveratrol for 48 h. Exponentially dividing cells were treated with increasing concentrations of resveratrol. Cell viability was determined using the MTT assay. The percentage of growth was calculated, with 100% representing control cells treated with 0.3% DMSO alone. The results are the means ± SDs from duplicate experiments (*, $P < 0.05$).

Lipofectin (Life Technologies, Inc.) with two plasmids, one encoding a cyclin D1 promoter-luciferase construct and the other encoding a cytomegalovirus promoter-β-galactosidase construct. The cyclin D1 construct (-1745CD1LUC) was generously provided by Dr. R. Pestell (Albert Einstein College of Medicine, Bronx, NY). Transfected cells were incubated overnight and then treated with resveratrol at a concentration of either 30, 100, or 300 μM. Cells were harvested after 6, 12, and 24 h of treatment and then analyzed for luciferase and β-galactosidase activities, as described previously (22). The β-galactosidase activities were used to correct for possible differences in transfection efficiency.

RT-PCR. SW480 cells were plated in 10-cm culture dishes at concentrations determined to yield 60–70% confluence within 24 h. Cells were treated with either DMSO (0.1%) or resveratrol (30, 100, and 300 μM). After a 12-h treatment, adherent cells were harvested, and total RNA was isolated using Trizol reagent (Life Technologies, Inc.) according to the manufacturer's instructions. Cyclin D1 and β-actin cDNAs were generated from 1 μg of total RNA using specific primers and the Superscript One-Step RT-PCR system with Platinum Taq (Life Technologies, Inc.). Sequences for cyclin D1-specific primers were as follows: CD13, 5′-GAACAAACAGATCATCCGCAA-3′; and CD14, 5′-TGCTCCTGGCAGGCACGGA-3′ (23). β-Actin-specific PCR products were amplified using specific primers (primer 1, 5′-CCAGGCACCAGGGCGTGATG-3′; primer 2, 5′-CGGCCAGCCAGGTCCAGACG-3′) and served as internal loading controls. PCR was conducted for 20–35 cycles in a Programmable Thermal Controller (MJ Research Inc., Watertown, MA). Each amplification cycle consisted of 0.5 min at 94°C for denaturation, 0.5 min at 55°C for primer annealing, and 1 min at 72°C for extension. After PCR amplification, the fragments were analyzed by agarose gel electrophoresis.

Statistical Analyses. Data are expressed as mean ± SD. Comparisons between DMSO-treated control cells and resveratrol-treated cells were made using Student's *t* test. Differences between groups of $P < 0.05$ were considered statistically significant.

RESULTS

Resveratrol Causes Dose-dependent Growth Inhibition in Several Human Cancer Cell Lines. To examine the antitumor activity of resveratrol in a variety of human cancer cell lines, we investigated its effects on cell growth in cell lines of different histological subtypes (HCE7 esophageal squamous carcinoma, Bic-1 esophageal adenocarcinoma, Seg-1 esophageal adenocarcinoma, SW480 colon adenocarcinoma, MCF7 breast adenocarcinoma, and HL60 promyelocytic leukemia cells). Exponentially dividing cells were treated with increasing concentrations of resveratrol (30–300 μM) for 48 h. In the Seg-1, HCE7, SW480, MCF7, and HL60 cell lines, resveratrol caused marked growth inhibition, in a dose-dependent fashion, with IC_{50} values in the range of 70–150 μM (Fig. 1). However, the Bic-1 cells were more resistant to growth inhibition because 100 μM resveratrol caused only about 20% growth inhibition (Fig. 1). Statistically significant reductions in cell viability were seen after treatment with 50 μM resveratrol in only three of the six cell lines (MCF7, HL60, and Seg-1), whereas all cell lines were significantly inhibited after treatment with 100 μM resveratrol (Fig. 1).

Resveratrol Induces Apoptosis in Several Human Cancer Cell Lines. In view of the above-mentioned growth-inhibitory effects, we were interested in determining whether resveratrol also induced apoptosis in these cell lines. The cells were treated with either DMSO alone or 300 μM resveratrol for 48 h. Because we were interested in simultaneously demonstrating growth inhibition, apoptosis, cell cycle arrest, and changes in biomarker expression in each cell line, we chose a single concentration of 300 μM for most of the subsequent assays. This dose is at least twice the IC_{50} value of each cell type. Repre-

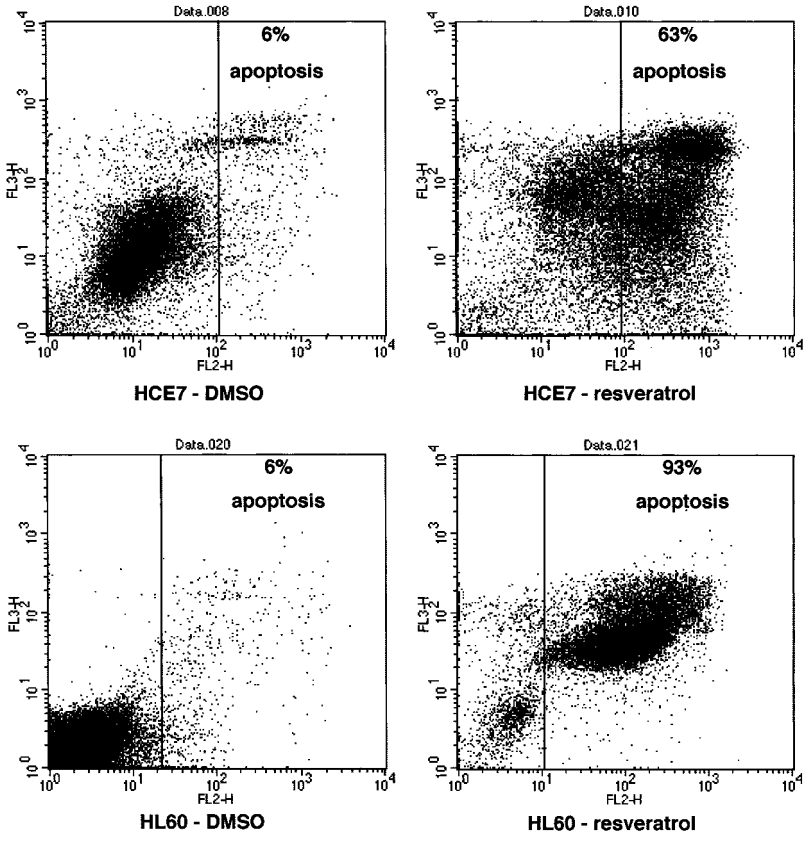

Fig. 2 Apoptosis induction. HCE7 and HL60 cells were treated with 0.3% DMSO or resveratrol (300 μM) for 48 h. Cells were double-stained with annexin V-PE and 7-aminoactinomycin and analyzed for apoptosis by DNA flow cytometry. The data indicate the percentage of annexin V-positive cells (apoptosis). All experiments were performed in duplicate and gave similar results.

Table 1 Apoptosis induction in human cancer cell lines after treatment for 48 h with DMSO or resveratrol (300 μM)

	% Apoptosis[a]	
Cell line	DMSO	Resveratrol
Bic-1	9	19
Seg-1	14	75
HCE7	6	63
MCF7	10	36
SW480	4	51
HL60	6	93

[a] Apoptosis was determined using an annexin V-based assay and flow cytometry (Fig. 2). The data indicate the percentage of cells undergoing apoptosis in each sample. All experiments were conducted in duplicate and gave similar results.

sentative results for the HCE7 and HL60 cells are shown in Fig. 2. In HCE7 cells, the percentage of apoptotic cells increased from 6% in control cells to 63% after treatment with resveratrol, and in the HL60 cells, the percentage of apoptotic cells increased from 6% to 93%, respectively. Data for the other cell lines are summarized in Table 1. Again, it is apparent that the Bic-1 cells are relatively resistant to resveratrol. Resveratrol (300 μM) also induced apoptosis in each of the cell lines after 24 h of treatment, although to a lesser extent (data not shown). Our results are consistent with those of previous studies indicating that resveratrol induces apoptosis in the HL60, T47D, A431, and JB6 cell lines (3, 5, 7).

Resveratrol Induces S-phase Cell Cycle Arrest in Several Human Cancer Cell Lines. We were also interested in examining the effects of resveratrol on cell cycle progression in exponentially dividing cultures of these cell lines. Subconfluent cultures of cells were treated with either DMSO alone or resveratrol (300 μM), at the same concentration used in the above-mentioned apoptosis assays. After 24 h of treatment, cells were labeled with PI and analyzed by DNA flow cytometry. Because we were interested in evaluating the distribution of actively dividing cells before the induction of extensive apoptosis, we harvested cells at 24 h, rather than at 48 h. A representative histogram for the HCE7 cells is shown in Fig. 3, and the data obtained with the other cell lines are summarized in Table 2. In the HCE7, MCF7, SW480, and HL60 cells, resveratrol caused an increase of cells in the S phase and a corresponding decrease of cells in the G_1 and G_2-M phases. In the Bic-1 cell line, there was an increase of cells in the G_1 phase, and in the Seg-1 cells, no significant change in cell cycle distribution was seen. Of note, in resveratrol-treated Seg-1 cells, 50% of the cells were undergoing apoptosis after only 24 h of treatment, as detected in a prominent sub-G_1 apoptotic peak (Table 2). Sub-G_1 apoptotic peaks were also detected in the remaining cell lines after 24 h of treatment (Table 2) and confirmed using the annexin V-based apoptosis assay (data not shown).

Resveratrol Causes a Decrease in the Expression Levels of Cyclins D1, A, and B1. Because of the effects on cell cycle progression seen in Fig. 3 and Table 2, we examined the effects

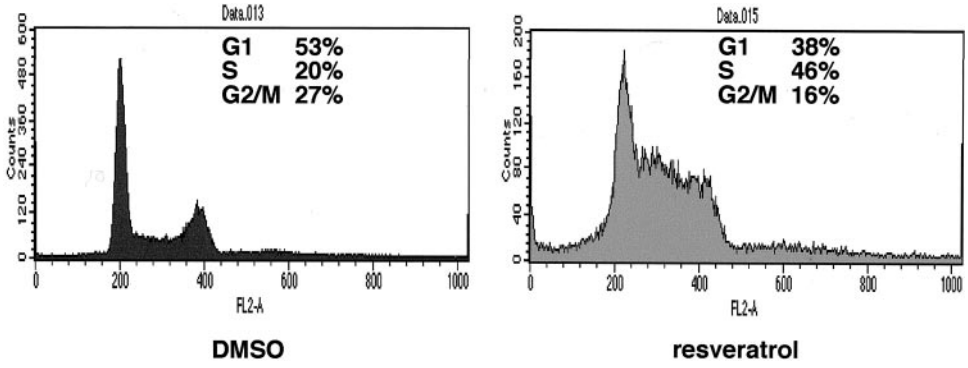

Fig. 3 Cell cycle analysis of HCE7 cells after treatment with 0.3% DMSO or 300 μM resveratrol. After 24 h of treatment, cells were labeled with PI and analyzed by DNA flow cytometry. The data indicate the percentage of cells in each phase of the cell cycle. All experiments were performed in duplicate and gave similar results.

Table 2 Cell cycle distribution of human cancer cell lines after treatment for 24 h with DMSO or resveratrol (300 μM)

		Distribution (% cells)[a]			
Cell line	Treatment	Sub-G_1	G_1	S	G_2-M
Bic-1	DMSO	1	55	26	19
	Resveratrol	4	61	28	11
Seg-1	DMSO	8	62	20	20
	Resveratrol	50	52	26	22
HCE7	DMSO	4	53	20	27
	Resveratrol	11	38	46	16
MCF7	DMSO	0	43	23	34
	Resveratrol	1	34	36	30
SW480	DMSO	4	50	37	13
	Resveratrol	15	35	50	15
HL60	DMSO	0	43	32	25
	Resveratrol	28	36	49	14

[a] DNA content was analyzed using PI staining and DNA flow cytometry (Fig. 3). The data indicate the percentage of cells in each phase of the cell cycle. All experiments were conducted in duplicate and gave similar results.

of resveratrol on the levels of cyclins in SW480 cells. We conducted a series of time course experiments using cellular protein extracts after 2, 6, 24, and 48 h of treatment with either 0.3% DMSO or 300 μM resveratrol (Fig. 4*A*). Untreated SW480 cells served as control cells. Western blot analysis demonstrated that cyclin A and cyclin B1 expression levels did not change initially but decreased after 24 and 48 h of treatment. Interestingly, cyclin D1 expression decreased after only 2 h of treatment and remained diminished after 24 and 48 h of treatment. A lower dose of resveratrol near the IC_{50} value (100 μM) also decreased cyclin D1 expression significantly at 2, 6, 24, and 48 h (Fig. 8; data not shown). Resveratrol treatment did not affect cyclin E expression (data not shown). Thus, although the predominant effect of resveratrol was to induce S-phase arrest, cyclin D1 appears to be the initial cyclin affected.

Because resveratrol induced an S-phase cell cycle arrest in the majority of cell lines tested, we investigated whether the G_2-phase cyclin, cyclin B1, was detectable and affected by resveratrol treatment. Cells were treated with 0.3% DMSO or 300 μM resveratrol and analyzed for levels of cyclin B1 expression after 48 h of treatment (Fig. 4*B*). Cyclin B1 expression was detectable in and decreased in all cell lines after treatment with resveratrol. Again, Bic-1 cells appeared to be less sensitive to resveratrol, and its effects on cyclin B1 were diminished. Thus, this fairly uniform decrease in cyclin B1 expression could explain resveratrol's ability to inhibit G_2-phase entry.

Resveratrol Decreases β-Catenin Expression in SW480 Cells and Induces Cox-2 Expression in Seg-1 Cells. β-Catenin is involved in both colon carcinogenesis and cyclin D1 transcriptional regulation (24, 25). Therefore, we investigated whether resveratrol induced changes in β-catenin expression in adenomatous polyposis coli gene-mutated SW480 cells, in which β-catenin is known to accumulate (25). Cells were treated with either 0.3% DMSO or 300 μM resveratrol. Cellular extracts were evaluated for β-catenin expression after 2, 6, 24, and 48 h of treatment. Western blot analysis demonstrated that β-catenin expression did not change initially but decreased after 24 and 48 h of treatment (Fig. 5*A*).

We also examined whether resveratrol affected cox-2 protein expression because of this protein's emerging role in both cancer development and chemoprevention. Cells were treated with 0.3% DMSO or 300 μM resveratrol and analyzed for levels of cox-2 expression after 48 h of treatment. Only Seg-1 cells expressed basal levels of cox-2, and this level was further induced by resveratrol (Fig. 5*B*). Neither basal levels nor induction of cox-2 was detectable in any of the other cell lines.

Resveratrol Does Not Decrease Cyclin D1 Promoter Activity. Because cyclin D1 expression was reduced by resveratrol after only 2 h of treatment, we investigated whether this was primarily a transcriptional or posttranslational event. Using a luciferase-based reporter assay (22), we investigated whether resveratrol treatment affects cyclin D1 promoter activity. We transfected SW480 cells with a plasmid containing a cyclin D1 promoter-luciferase construct. Cells were cotransfected with a plasmid containing a cytomegalovirus promoter-β-galactosidase construct to serve as an internal control and account for differences in transfection efficiency. After transfection, we incubated these cells with resveratrol (30, 100, and 300 μM) for 6, 12, and 24 h. Luciferase activity was not significantly diminished by resveratrol after 6 and 12 h of treatment (Fig. 6; 6 h time points not shown). In fact, there was even a slight induction in activity after 12 h of treatment. Therefore, these results suggest that the decrease in cyclin D1 protein expression induced by resveratrol is not due to a reduction in cyclin D1 transcription. Note that because a large proportion of transfected cells treated at the higher dose (300 μM) were floating, they were not collected during the assay, which determines activity only in the adherent

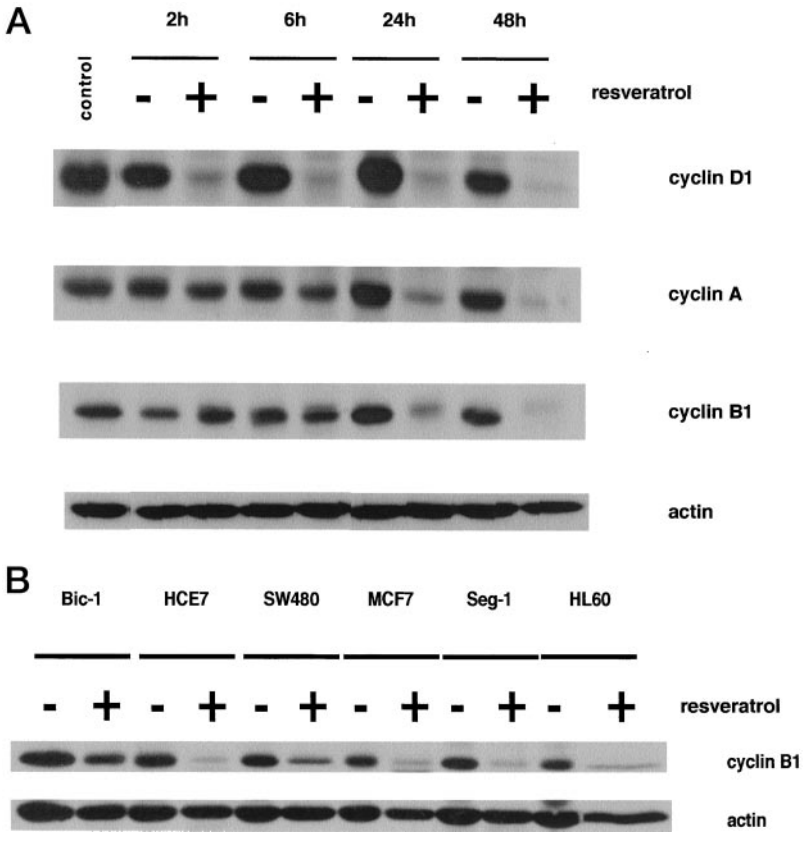

Fig. 4 *A,* Cyclin D1, cyclin A, and cyclin B1 protein expression in SW480 cells. Time course experiments in which cells were treated with 0.3% DMSO (−) or 300 μM resveratrol (+) were performed. Control cells were untreated. After 2, 6, 24, and 48 h of treatment, cell lysates were evaluated for levels of cyclin D1, cyclin A, and cyclin B1 expression by Western blotting as described in "Materials and Methods." *B,* Cyclin B1 protein expression in six human cancer cell lines after treatment with 0.3% DMSO (−) or 300 μM resveratrol (+). After 48 h of treatment, cell lysates were evaluated for cyclin B1 expression by Western blotting.

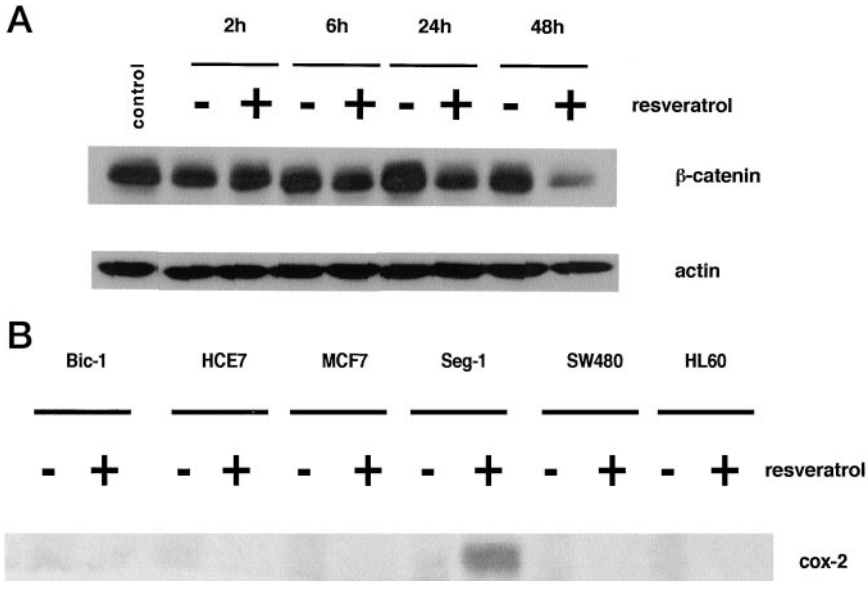

Fig. 5 *A,* β-catenin protein expression in SW480 cells. A time course experiment in which cells were treated with 0.3% DMSO (−) or 300 μM resveratrol (+) was performed. Control cells were untreated. After 2, 6, 24, and 48 h of treatment, cell lysates were evaluated for levels of β-catenin expression by Western blotting. *B,* Cox-2 protein expression in six human cancer cell lines after treatment with 0.3% DMSO (−) or 300 μM resveratrol (+). After 48 h of treatment, cell lysates were evaluated for cox-2 expression by Western blotting.

cells. Similar gross cellular toxicity was seen in the 24-h-treated samples (data not shown).

High Doses of Resveratrol Decrease Cyclin D1 mRNA Levels. The reduction in cyclin D1 protein expression induced by resveratrol was not accompanied by a reduction in cyclin D1 promoter activity. We further examined whether the levels of cyclin D1 mRNA were affected by resveratrol treatment using a semiquantitative RT-PCR-based assay. In this assay, PCR products are generated during both plateau and log-phase reactions by conducting 20-, 25-, and 35-cycle rounds of PCR. In this

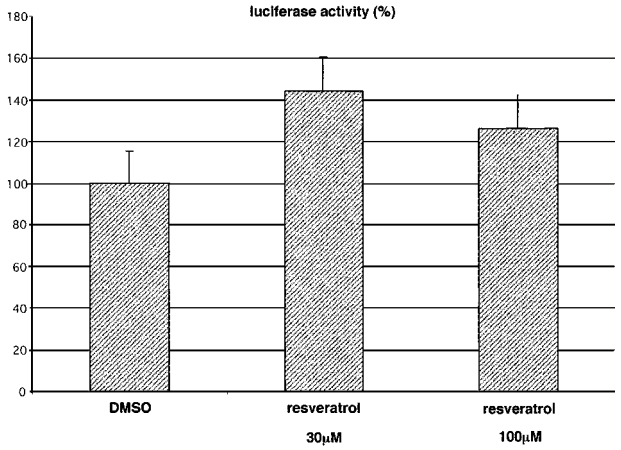

Fig. 6 Cyclin D1 promoter activity in SW480 cells after treatment with DMSO or resveratrol for 12 h. Cells were transfected with a cyclin D1-luciferase reporter construct, as described in "Materials and Methods." The data are expressed as the percentage of luciferase activity, with 100% activity resulting from DMSO-treated cells. The results are the means ± SDs from duplicate experiments.

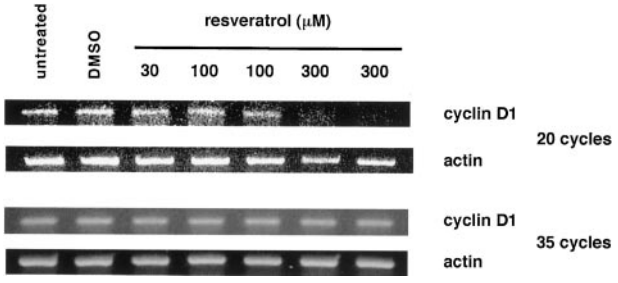

Fig. 7 RT-PCR analysis of cyclin D1 mRNA transcripts. RNA was isolated from untreated SW480 cells or from SW480 cells treated with DMSO or resveratrol.

approach, these products have been shown to reflect corresponding levels of mRNA (26). In Fig. 7, cyclin D1 band intensities were similar in untreated cells and in cells treated with DMSO and 30–100 μM resveratrol after 20-cycle rounds of PCR. However, cyclin D1 products were not detected after SW480 cells were treated with 300 μM resveratrol. Of note, several culture dishes of cells treated at this dose were harvested, given the significant cellular toxicity induced by higher doses of resveratrol. In contrast, cyclin D1 band intensities of the 25- and 35-cycle PCR products were unchanged in cells treated at each dose level of resveratrol (25-cycle round PCR products not shown). These results suggest that at doses greater than its IC_{50} value, resveratrol appears to diminish the level of cyclin D1 mRNA. Thus, higher doses of resveratrol may contribute to decreased cyclin D1 protein expression by negatively regulating transcription.

Resveratrol Induces Cyclin D1 Degradation. Because resveratrol is able to reduce cyclin D1 protein expression apparently without affecting transcription, we investigated whether this reduction is due to posttranslational changes. Recent studies have proposed cyclin D1 proteolysis as a chemoprevention signal and mechanism of action of retinoid compounds (27–29). In these reports, cyclin D1 degradation was inhibited by the proteasome inhibitor LLnL. We investigated whether LLnL could similarly prevent the decrease in cyclin D1 expression induced by resveratrol. SW480 cells were treated with DMSO or resveratrol (100 or 300 μM) or cotreated with resveratrol plus LLnL (100 μM). Because the 300 μM dose exceeded its IC_{50} value, we included the 100 μM dose level to confirm resveratrol's efficacy in decreasing cyclin D1 expression at doses closer to its IC_{50} value. After 24 h of treatment, cells were harvested, and protein lysates were prepared. Of note, there was a large amount of floating cells observed in those plates cotreated with the LLnL compound. Western blot analysis demonstrated that cyclin D1 expression was highest in DMSO-treated cells, slightly reduced in cells cotreated with both compounds, and significantly reduced in cells treated with resveratrol alone (Fig. 8). Therefore, these experiments demonstrate that LLnL is able to partially inhibit the negative effect of resveratrol on cyclin D1 expression. Thus, the early reduction in cyclin D1 expression induced by resveratrol may be due to cyclin D1 degradation.

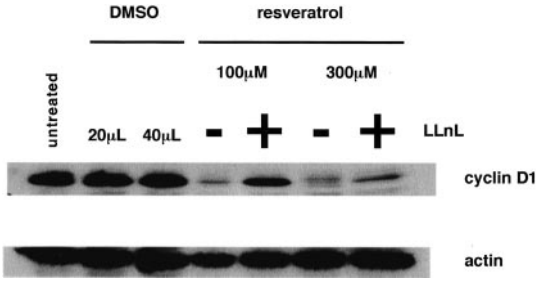

Fig. 8 Cyclin D1 protein expression in SW480 cells after cotreatment with resveratrol and LLnL. Cells were treated with 0.3% DMSO or resveratrol (100 or 300 μM) with or without LLnL (100 μM). After 24 h of treatment, cell lysates were evaluated for cyclin D1 expression by Western blotting.

DISCUSSION

Resveratrol is currently being evaluated in preclinical studies as a potential cancer chemoprevention agent. It has previously been shown to have anticancer activities in both cell culture and animal carcinogenesis models of both hematological and solid tumors. Although it is widely available in the form of unregulated herbal supplements, there are relatively little clinical data characterizing its anticancer activities during human consumption. We carried out the present studies to provide further evidence to support the use of this compound in cancer prevention and therapy trials and to identify a panel of surrogate biomarkers for evaluating its *in vivo* treatment efficacy. Our studies demonstrate the broad antitumor properties of this agent in a wide variety of human cancer cell lines. Resveratrol caused a dose-dependent cancer cell growth inhibition, and this antiproliferative effect appears to be due to its ability to induce S-phase arrest and apoptotic cell death. Furthermore, we have identified cyclin D1, cyclin A, cyclin B1, β-catenin, apoptotic index, S-phase arrest, and possibly cox-2 as candidate biomarkers for use as surrogate intermediate end points. We have

further characterized resveratrol's mechanism of action in SW480 human colorectal cancer cells. In these cells, resveratrol decreases the expression levels of cyclin D1, cyclin A, cyclin B1, and β-catenin. The decrease in cyclin D1 expression appears to be due more to an induction of its degradation than to a suppression of its transcription.

Resveratrol has been previously shown to have growth-inhibitory activity in several human cancer cell lines of both hematological and epithelial origin, including HL60 leukemia (3), CaCo-2 colorectal carcinoma (6), and A431 epidermoid carcinoma cells (7). In breast cancer cell lines, however, its effects on cell growth were not consistent. At higher doses (≥50 μM), resveratrol generally inhibited cell growth in both ER+ and ER− breast cancer cell lines (9, 11), although one study reported growth inhibition in the 22–175 μM dose range (8). At lower doses (≤25 μM), resveratrol stimulated cell growth in ER+ breast cancer cells (9–11). Structurally, resveratrol resembles the synthetic estrogen diethylstilbesterol (12) and can bind to rat uterine ERs (12, 30), although at a much lower affinity than estradiol. Resveratrol has also been shown to activate transcription of estrogen-responsive reporter constructs (10, 31). However, when given s.c. to Wistar rats, resveratrol failed to induce significant uterotrophic responses, suggesting that its potential estrogenic activity may not be relevant in *in vivo* models (12, 30). However, because of its estrogenic potential, caution should be used when evaluating its clinical role in breast cancer therapy and prevention.

Resveratrol has also been previously shown to induce apoptosis in leukemia, mammary, and epidermoid cell lines (3, 5, 7). The doses of resveratrol used to induce cellular changes, including growth inhibition, cell cycle arrest, and apoptosis, can be divided into three different dose ranges. Whereas resveratrol can induce specific biochemical effects in cell culture models in the 1–10 μM range, its cytostatic and cytotoxic effects usually require 25–100 and 100–200 μM concentrations, respectively. Previous investigators have demonstrated resveratrol's abilities to decrease cyclin D1 expression (7, 32), reduce [^{3}H]thymidine incorporation (33), inhibit phorbol ester-mediated cox-2 induction (34), decrease ornithine decarboxylase activity (6), and reduce indices of oxidative damage (35) at concentrations in the 10–30 μM range. Two previous studies evaluated concentrations in the 1–10 μM range (3, 7). In both studies, however, the majority of resveratrol-induced effects, including significant growth inhibition, occurred only at concentrations above 25 μM. Thus, the latter and other studies have shown that doses of resveratrol in the range of 25–100 μM are required to inhibit growth in various human cancer and leukemia cell lines (3, 4, 6, 7, 9, 32, 35) and that treatment with concentrations below this range had little effect on growth (4, 6). Similar doses were also able to induce cell cycle arrest (3, 6, 7, 32, 35, 36). Two previous studies demonstrated resveratrol's ability to induce apoptosis at its IC_{50} dose for growth inhibition (3, 7). However, in most of the previous studies, resveratrol did not induce significant apoptosis or have cytotoxic effects at cytostatic doses (6, 32, 35, 36). Thus, doses required for resveratrol to induce apoptosis were often higher than those that induced growth inhibition and cell cycle arrest (4) and were often in the 100–200 μM range (32, 37, 38). In the present study, we chose a dose of 300 μM to convincingly demonstrate the ability of resveratrol to induce apoptosis in a variety of human cancer cell lines, including esophageal and colorectal carcinoma cells, types of cancer in which resveratrol may have a role in chemoprevention.

As with other types of chemoprevention agents, including nonsteroidal anti-inflammatory drugs and retinoid compounds, the antitumor and antiproliferative activities of resveratrol probably reflect several mechanisms of action. In the present studies, growth inhibition and induction of apoptosis were observed within 48 h of treatment. A slight amount of apoptosis could be detected after only 24 h of treatment by flow cytometry using an annexin V-based staining assay (data not shown). After only 24 h of treatment, resveratrol prevented cells from entering the G_2 phase of the cell cycle, resulting in the accumulation of cells in either the G_1 or S phase. Most of the cell lines demonstrated an accumulation in S phase, but in Bic-1 cells, resveratrol treatment led to G_1-phase arrest (Table 2). Resveratrol did not appear to alter the cell cycle distribution in Seg-1 cells, perhaps because of the extensive apoptosis (50%) seen after only 24 h of treatment (Table 2). The ability of resveratrol to block the S-G_2 transition has been reported previously in HL60 leukemia (4), U937 lymphoma (36), and CaCo-2 colon cancer cells (6). However, other investigators have reported an arrest in the G_1 phase with A431 cells (7). In the Yoshida rat hepatoma model, Carbo *et al.* (14) demonstrated a G_2-M-phase cell cycle arrest. The latter authors suggested that in their *in vivo* model, lower cellular proliferation rates and host factors, including immune system-mediated events, might explain this difference. Therefore, the effects of resveratrol on cell cycle progression can vary in different experimental systems.

To further characterize the effects of resveratrol, we examined by Western blot analysis the levels of expression of several proteins after treating SW480 colon carcinoma cells with resveratrol. We found that cyclin B1, cyclin A, and β-catenin expression levels were decreased after 24 and 48 h of treatment. Cyclin D1 expression decreased within 2 h of treatment and remained diminished at all subsequent time points (up to 48 h). In previous studies of colon cancer cell lines, resveratrol induced growth inhibition and S-G_2 transition arrest but did not induce apoptosis in CaCo-2 cells (6). This may be because Schneider *et al.* (6) used a lower dose (25 μM) of the drug. The latter authors did not investigate the effects of resveratrol on cell cycle kinetics or on the expression of cyclins. Resveratrol was recently shown to induce growth inhibition in CaCo-2 cells in the 12.5–200 μM range, but 200 μM resveratrol was required to induce apoptosis (32). These dose effects are similar to those used in our studies. Wolter *et al.* (32) also demonstrated that resveratrol decreased cyclin D1 expression.

The effects of resveratrol on cell cycle control proteins have been studied previously in other cell types, but the findings have not been uniform. In a study of HL60 cells, resveratrol increased the levels of cyclin A and cyclin E but did not affect the G_1-phase proteins, cyclin D1, p21, p27, cdk2, or cdk4/6 (4). In this report, HL60 leukemia cells accumulated in the S phase. In A431 epidermoid cancer cells, however, resveratrol decreased the levels of cyclin D1, cyclin D2, cyclin E, cdk2, and cdk4/6, and these cells were arrested in the G_1 phase (7). In U937 lymphoma cells, resveratrol increased the levels of cyclin E, cyclin A, and cyclin D3, decreased the level of cdk2, and did not affect the levels of cyclin B1, cdk4, or cdc2, although the

cells were arrested in S phase (36). Therefore, the effects of resveratrol on the expression of cell cycle control proteins appear to also vary considerably between cell systems.

As mentioned above, in the present study, resveratrol caused a rapid and sustained decrease in cyclin D1 expression in SW480 cells. Because this decrease was inhibited by the proteasome inhibitor LLnL (Fig. 8), it appears to be due primarily to degradation of the cyclin D1 protein. The results we obtained in cyclin D1 promoter-luciferase reporter assays (Fig. 6) and in studies of cyclin D1 mRNA (Fig. 7) are consistent with this conclusion, at least for doses near resveratrol's IC_{50} value. However, at higher doses, resveratrol appears to decrease cyclin D1 protein expression both by inducing its degradation and by causing a decrease in cyclin D1 mRNA (Figs. 7 and 8). It is of interest that treatment of bronchial epithelial and embryonal carcinoma cell lines with all-*trans*-retinoic acid also led to degradation of the cyclin D1 protein (27–29). Rapid cyclin D1 proteolysis has also been observed as a cellular response to generalized DNA damage (39). It is curious, however, that despite this rapid decrease in cyclin D1 in the present studies, the resveratrol-treated cells arrested in the S phase rather than in the G_1 phase. We found that in SW480 cells, resveratrol had no effect on cyclin E (data not shown), which also regulates progression through the G_1-S transition (36). Perhaps, despite the decrease in cyclin D1, the continued cyclin E expression allows the cells to enter the S phase. In fact, in previous studies, whereas decreased cyclin D1 expression correlated with G_1-phase arrest (7, 39), increased cyclin E expression correlated with S-phase arrest (4, 36). Our finding that after 24 h there was a decrease in cyclin B1 (Fig. 4*B*) is consistent with the S-phase arrest. Some authors have suggested that resveratrol induces S-phase arrest by decreasing the rate of DNA synthesis (4, 6). In fact, resveratrol has been shown to inhibit ribonucleotide reductase activity in murine leukemia L1210 cells (33), to inhibit DNA synthesis as measured by [^{3}H]thymidine incorporation in murine mastocytoma P-815 cells and human leukemia K-562 cells (33), and to possibly inhibit DNA polymerase in SV40-infected cells (40). Therefore, there are several plausible mechanisms for the arrest in S phase induced by resveratrol.

It is of interest that the Bic-1 human esophageal adenocarcinoma cell line was relatively resistant to resveratrol with respect to growth inhibition (Fig. 1), cell cycle arrest (Table 2), apoptosis (Table 1), and altered expression of cyclin B1 (Fig. 4*B*). This cell line may therefore be useful for additional studies of the mechanism of action of resveratrol and may thereby be useful for predicting which human tumors might not respond to therapy with resveratrol.

There is considerable current interest in the use of selective cox-2 inhibitors as chemoprevention agents (41). Previous studies with resveratrol have given conflicting results. Although some cell culture studies have demonstrated inhibition of cox-2 expression and activity (34, 42), other studies with resveratrol have shown no effect on cox-2 activity or have found stimulation of cox-2 activity in cell-free biochemical assays (1, 43). In the present studies, the Seg-1 cells expressed a basal level of the cox-2 protein, and, surprisingly, the level was induced after treatment with resveratrol (Fig. 5*B*). The reason for this induction is not clear but may represent a generalized cellular stress reaction. On the other hand, we did not detect cox-2 expression in any of the remaining cell lines, either at baseline or after treatment with resveratrol (Fig. 5*B*), yet the majority of these cell lines demonstrated similar sensitivity to the antiproliferative effects of resveratrol. These findings provide evidence that cox-2 is not the critical target for the antitumor activity of this compound.

There is limited information on the toxicity of resveratrol in experimental animals, and there are, apparently, no clinical toxicity data on the use of pure resveratrol in humans. In the present study, we used fairly high doses of resveratrol, although resveratrol's effects on cyclin D1 were also seen with the IC_{50} dose (Fig. 8). Previous studies have demonstrated that resveratrol is minimally toxic to human peripheral blood cells (3). The clinical implications of our studies will depend on whether resveratrol can be given safely to humans at doses high enough to achieve pharmacologically active levels. Due to its polyphenolic nature, resveratrol could conceivably accumulate in tumor tissue at levels that exceed its concentration in the serum. *In vivo* tissue levels of resveratrol have not been reported, but in pharmacokinetic studies, Soleas *et al.* (44) demonstrated that 50–75% of the administered dose of tritiated resveratrol was absorbed by rats after oral administration, yet the blood and plasma levels were scarcely above background. Because of resveratrol's high lipid solubility, the authors postulated that the compound was deposited in adipose tissue and other tissues with high lipid content, but they did not actually measure tissue levels. Using gas chromatography, these authors also demonstrated a 10–15% absorption rate in humans after consumption of a 25 mg/100 ml preparation of resveratrol in wine (45). Our findings also suggest that assays for cyclin D1, cyclin B1, β-catenin or apoptosis in tumor biopsy samples might provide useful surrogate end points in clinical therapy trials.

ACKNOWLEDGMENTS

We thank Windsor Ting, Elina Korkin, and Mehmet Oz from Columbia University and Bolin Zheng and Qun Yi Zheng from PureWorld Botanicals for assistance with this project.

REFERENCES

1. Jang, M., Cai, L., Udeani, G. O., Slowing, K. V., Thomas, C. F., Beecher, C. W., Fong, H. H., Farnsworth, N. R., Kinghorn, A. D., Mehta, R. G., Moon, R. C., and Pezzuto, J. M. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. Science (Wash. DC), *275:* 218–220, 1997.

2. Soleas, G. J., Diamandis, E. P., and Goldberg, D. M. Resveratrol: a molecule whose time has come? And gone? Clin. Biochem., *30:* 91–113, 1997.

3. Clement, M. V., Hirpara, J. L., Chawdhury, S. H., and Pervaiz, S. Chemopreventive agent resveratrol, a natural product derived from grapes, triggers CD95 signaling-dependent apoptosis in human tumor cells. Blood, *92:* 996–1002, 1998.

4. Ragione, F. D., Cucciolla, V., Borriello, A., Pietra, V. D., Racioppi, L., Soldati, G., Manna, C., Galletti, P., and Zappia, V. Resveratrol arrests the cell division cycle at S/G_2 phase transition. Biochem. Biophys. Res. Commun., *250:* 53–58, 1998.

5. Huang, C., Ma, W. Y., Goranson, A., and Dong, Z. Resveratrol suppresses cell transformation and induces apoptosis through a p53-dependent pathway. Carcinogenesis (Lond.), *20:* 237–242, 1999.

6. Schneider, Y., Vincent, F., Duranton, B., Badolo, L., Gosse, F., Bergmann, C., Seiler, N., and Raul, F. Anti-proliferative effect of resveratrol, a natural component of grapes and wine, on human colonic cancer cells. Cancer Lett., *158:* 85–91, 2000.

7. Ahmad, N., Adhami, V. M., Afaq, F., Feyes, D. K., and Mukhtar, H. Resveratrol causes waf-1/p21-mediated G_1-phase arrest of cell cycle and induction of apoptosis in human epidermoid carcinoma A431 cells. Clin. Cancer Res., *7:* 1466–1473, 2001.

8. Mgbonyebi, O. P., Russo, J., and Russo, I. H. Antiproliferative effect of synthetic resveratrol on human breast epithelial cells. Int. J. Oncol., *12:* 865–869, 1998.

9. Nakagawa, H., Kiyozuka, Y., Uemura, Y., Senzaki, H., Shikata, N., Hioki, K., and Tsubura, A. Resveratrol inhibits human breast cancer cell growth and may mitigate the effect of linoleic acid, a potent breast cancer cell stimulator. J. Cancer Res. Clin. Oncol., *127:* 258–264, 2001.

10. Gehm, B. D., McAndrews, J. M., Chien, P. Y., and Jameson, J. L. Resveratrol, a polyphenolic compound found in grapes and wine, is an agonist for the estrogen receptor. Proc. Natl. Acad. Sci. USA, *94:* 14138–14143, 1997.

11. Basly, J. P., Marre-Fournier, F., Le Bail, J. C., Habrioux, G., and Chulia, A. J. Estrogenic/antiestrogenic and scavenging properties of (E)- and (Z)-resveratrol. Life Sci., *66:* 769–777, 2000.

12. Freyberger, A., Hartmann, E., Hildebrand, H., and Krotlinger, F. Differential response of immature rat uterine tissue to ethinylestradiol and the red wine constituent resveratrol. Arch. Toxicol., *74:* 709–715, 2001.

13. Tessitore, L., Davit, A., Sarotto, I., and Caderni, G. Resveratrol depresses the growth of colorectal aberrant crypt foci by affecting bax and $p21^{CIP}$ expression. Carcinogenesis (Lond.), *21:* 1619–1622, 2000.

14. Carbo, N., Costelli, P., Baccino, F. M., Lopez-Soriano, F. J., and Argiles, J. M. Resveratrol, a natural product present in wine, decreases tumour growth in a rat tumour model. Biochem. Biophys. Res. Commun., *254:* 739–743, 1999.

15. Goldberg, D. M., Garovic-Kocic, V., Diamandis, E. P., and Pace-Asciak, C. R. Wine: does the colour count? Clin. Chim. Acta, *246:* 183–193, 1996.

16. Pace-Asciak, C. R., Rounova, O., Hahn, S. E., Diamandis, E. P., and Goldberg, D. M. Wines and grape juices as modulators of platelet aggregation in healthy human subjects. Clin. Chim. Acta, *246:* 163–182, 1996.

17. Banks-Schlegel, S. P., and Quintero, J. Growth and differentiation of human esophageal carcinoma cell lines. Cancer Res., *46:* 250–258, 1986.

18. Jiang, W., Zhang, Y. J., Kahn, S. M., Hollstein, M. C., Santella, R. M., Lu, S. H., Harris, C. C., Montesano, R., and Weinstein, I. B. Altered expression of the cyclin D1 and retinoblastoma genes in human esophageal cancer. Proc. Natl. Acad. Sci. USA, *90:* 9026–9030, 1993.

19. Vermes, I., Haanen, C., Steffens-Nakken, H., and Reutelingsperger, C. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labeled annexin V. J. Immunol. Methods, *184:* 39–51, 1995.

20. Lim, J. T., Piazza, G. A., Han, E. K., Delohery, T. M., Li, H., Finn, T. S., Buttyan, R., Yamamoto, H., Sperl, G. J., Brendel, K., Gross, P. H., Pamukcu, R., and Weinstein, I. B. Sulindac derivatives inhibit growth and induce apoptosis in human prostate cancer cell lines. Biochem. Pharmacol., *58:* 1097–1107, 1999.

21. Shirin, H., Sordillo, E. M., Oh, S. H., Yamamoto, H., Delohery, T., Weinstein, I. B., and Moss, S. F. *Helicobacter pylori* inhibits the G_1 to S transition in AGS gastric epithelial cells. Cancer Res., *59:* 2277–2281, 1999.

22. Albanese, C., Johnson, J., Watanabe, G., Eklund, N., Vu, D., Arnold, A., and Pestell, R. G. Transforming $p21^{ras}$ mutants and c-Ets-2 activate the cyclin D1 promoter through distinguishable regions. J. Biol. Chem., *270:* 23589–23597, 1995.

23. Hosokawa, Y., and Arnold, A. Mechanism of cyclin D1 (CCND1, PRAD1) overexpression in human cancer cells: analysis of allele-specific expression. Genes Chromosomes Cancer, *22:* 66–71, 1998.

24. Behrens, J. Control of β-catenin signaling in tumor development. Ann. N. Y. Acad. Sci., *910:* 21–33; discussion, 33–35; 2000.

25. Wakita, K., Tetsu, O., and McCormick, F. A mammalian two-hybrid system for adenomatous polyposis coli-mutated colon cancer therapeutics. Cancer Res., *61:* 854–858, 2001.

26. Yoshimi, N., Ino, N., Suzui, M., Tanaka, T., Nakashima, S., Nakamura, M., Nozawa, Y., and Mori, H. The mRNA overexpression of inflammatory enzymes, phospholipase A2 and cyclooxygenase, in the large bowel mucosa and neoplasms of F344 rats treated with naturally occurring carcinogen, 1-hydroxyanthraquinone. Cancer Lett., *97:* 75–82, 1995.

27. Langenfeld, J., Kiyokawa, H., Sekula, D., Boyle, J., and Dmitrovsky, E. Posttranslational regulation of cyclin D1 by retinoic acid: a chemoprevention mechanism. Proc. Natl. Acad. Sci. USA, *94:* 12070–12074, 1997.

28. Boyle, J. O., Langenfeld, J., Lonardo, F., Sekula, D., Reczek, P., Rusch, V., Dawson, M. I., and Dmitrovsky, E. Cyclin D1 proteolysis: a retinoid chemoprevention signal in normal, immortalized, and transformed human bronchial epithelial cells. J. Natl. Cancer Inst. (Bethesda), *91:* 373–379, 1999.

29. Spinella, M. J., Freemantle, S. J., Sekula, D., Chang, J. H., Christie, A. J., and Dmitrovsky, E. Retinoic acid promotes ubiquitination and proteolysis of cyclin D1 during induced tumor cell differentiation. J. Biol. Chem., *274:* 22013–22018, 1999.

30. Ashby, J., Tinwell, H., Pennie, W., Brooks, A. N., Lefevre, P. A., Beresford, N., and Sumpter, J. P. Partial and weak oestrogenicity of the red wine constituent resveratrol: consideration of its superagonist activity in MCF-7 cells and its suggested cardiovascular protective effects. J. Appl. Toxicol., *19:* 39–45, 1999.

31. Bowers, J. L., Tyulmenkov, V. V., Jernigan, S. C., and Klinge, C. M. Resveratrol acts as a mixed agonist/antagonist for estrogen receptors α and β. Endocrinology, *141:* 3657–3667, 2000.

32. Wolter, F., Akoglu, B., Clausnitzer, A., and Stein, J. Downregulation of the cyclin D1/Cdk4 complex occurs during resveratrol-induced cell cycle arrest in colon cancer cell lines. J. Nutr., *131:* 2197–2203, 2001.

33. Fontecave, M., Lepoivre, M., Elleingand, E., Gerez, C., and Guittet, O. Resveratrol, a remarkable inhibitor of ribonucleotide reductase. FEBS Lett., *421:* 277–279, 1998.

34. Subbaramaiah, K., Chung, W. J., Michaluart, P., Telang, N., Tanabe, T., Inoue, H., Jang, M., Pezzuto, J. M., and Dannenberg, A. J. Resveratrol inhibits cyclooxygenase-2 transcription and activity in phorbol ester-treated human mammary epithelial cells. J. Biol. Chem., *273:* 21875–21882, 1998.

35. Sgambato, A., Ardito, R., Faraglia, B., Boninsegna, A., Wolf, F. I., and Cittadini, A. Resveratrol, a natural phenolic compound, inhibits cell proliferation and prevents oxidative DNA damage. Mutat. Res., *496:* 171–180, 2001.

36. Park, J. W., Choi, Y. J., Jang, M. A., Lee, Y. S., Jun, D. Y., Suh, S. I., Baek, W. K., Suh, M. H., Jin, I. N., and Kwon, T. K. Chemopreventive agent resveratrol, a natural product derived from grapes, reversibly inhibits progression through S and G_2 phases of the cell cycle in U937 cells. Cancer Lett., *163:* 43–49, 2001.

37. Mahyar-Roemer, M., and Roemer, K. $p21^{Waf1/Cip1}$ can protect human colon carcinoma cells against p53-dependent and p53-independent apoptosis induced by natural chemopreventive and therapeutic agents. Oncogene, *20:* 3387–3398, 2001.

38. Tinhofer, I., Bernhard, D., Senfter, M., Anether, G., Loeffler, M., Kroemer, G., Kofler, R., Csordas, A., and Greil, R. Resveratrol, a tumor-suppressive compound from grapes, induces apoptosis via a novel mitochondrial pathway controlled by Bcl-2. FASEB J., *15:* 1613–1615, 2001.

39. Agami, R., and Bernards, R. Distinct initiation and maintenance mechanisms cooperate to induce G_1 cell cycle arrest in response to DNA damage. Cell, *102:* 55–66, 2000.

40. Sun, N. J., Woo, S. H., Cassady, J. M., and Snapka, R. M. DNA polymerase and topoisomerase II inhibitors from *Psoralea corylifolia*. J. Nat. Prod. (Lloydia), *61:* 362–366, 1998.

41. Subbaramaiah, K., Zakim, D., Weksler, B. B., and Dannenberg, A. J. Inhibition of cyclooxygenase: a novel approach to cancer prevention. Proc. Soc. Exp. Biol. Med., *216:* 201–210, 1997.

42. Mutoh, M., Takahashi, M., Fukuda, K., Matsushima-Hibiya, Y., Mutoh, H., Sugimura, T., and Wakabayashi, K. Suppression of cyclooxygenase-2 promoter-dependent transcriptional activity in colon cancer cells by chemopreventive agents with a resorcin-type structure. Carcinogenesis (Lond.), *21:* 959–963, 2000.

43. Johnson, J. L., and Maddipati, K. R. Paradoxical effects of resveratrol on the two prostaglandin H synthases. Prostaglandins Other Lipid Mediat., *56:* 131–143, 1998.

44. Soleas, G. J., Angelini, M., Grass, L., Diamandis, E. P., and Goldberg, D. M. Absorption of *trans*-resveratrol in rats. Methods Enzymol., *335:* 145–154, 2001.

45. Soleas, G. J., Yan, J., and Goldberg, D. M. Measurement of *trans*-resveratrol, (+)-catechin, and quercetin in rat and human blood and urine by gas chromatography with mass selective detection. Methods Enzymol., *335:* 130–145, 2001.

Clinical Cancer Research

Resveratrol Induces Growth Inhibition, S-phase Arrest, Apoptosis, and Changes in Biomarker Expression in Several Human Cancer Cell Lines

Andrew K. Joe, Hui Liu, Masumi Suzui, et al.

Clin Cancer Res 2002;8:893-903.