Resveratrol modifies the expression of apoptotic regulatory proteins and sensitizes non-Hodgkin's lymphoma and multiple myeloma cell lines to paclitaxel-induced apoptosis

Ali R. Jazirehi and Benjamin Bonavida

Department of Microbiology, Immunology and Molecular Genetics and Jonsson Comprehensive Cancer Center, David Geffen School of Medicine, University of California at Los Angeles, Los Angeles, CA

Abstract

Resveratrol (*trans*-3,4,5-trihydroxystilbene) has received attention for its potential chemopreventive and antitumor effects in experimental systems. Recent evidence suggests that paclitaxel, alone or in combination with other drugs, can be effectively used in the treatment of non-Hodgkin's lymphoma (NHL) and multiple myeloma (MM). This study investigated whether resveratrol can sensitize NHL and MM cell lines to paclitaxel-mediated apoptosis and to delineate the underlying molecular mechanism of sensitization. Both resveratrol and paclitaxel negatively modulated tumor cell growth by arresting the cells at the G_2-M phase of the cell cycle. Low concentrations of resveratrol exerted a sensitizing effect on drug-refractory NHL and MM cells to apoptosis induced by paclitaxel. Resveratrol selectively down-regulated the expression of antiapoptotic proteins Bcl-x_L and myeloid cell differentiation factor-1 (Mcl-1) and up-regulated the expression of proapoptotic proteins Bax and apoptosis protease activating factor-1 (Apaf-1). Paclitaxel down-regulated the expression of Bcl-x_L, Mcl-1, and cellular inhibitor of apoptosis protein-1 antiapoptotic proteins and up-regulated Bid and Apaf-1. Combination treatment resulted in apoptosis through the formation of tBid, mitochondrial membrane depolarization, cytosolic release of cytochrome *c* and Smac/DIABLO, activation of the caspase cascade, and cleavage of poly(adenosine diphosphate-ribose) polymerase. Combination of resveratrol with paclitaxel had minimal cytotoxicity against quiescent and mitogenically stimulated human peripheral blood mononuclear cells. Inhibition of Bcl-x_L expression by resveratrol was critical for chemosensitization and its functional impairment mimics resveratrol-mediated sensitization to paclitaxel-induced apoptosis. Inhibition of Bcl-x_L expression by resveratrol was due to the inhibition of the extracellular signal-regulated kinase 1/2 (ERK1/2) pathway and diminished activator protein-1-dependent Bcl-x_L expression. The findings by resveratrol were corroborated with inhibitors of the ERK1/2 pathway. This study demonstrates that in resistant NHL and MM cell lines resveratrol and paclitaxel selectively modify the expression of regulatory proteins in the apoptotic signaling pathway and the combination, via functional complementation, results in synergistic apoptotic activity. [Mol Cancer Ther. 2004;3(1):71–84]

Introduction

The continuing search for cancer chemopreventive agents has resulted in the identification and characterization of various compounds that could potentially block the initiation of tumor formation. Recently, the plant polyphenol resveratrol (*trans*-3,4′,5-trihydroxystilbene) that is found in wine, grape, and peanut has been the focus of numerous research investigations due to its chemopreventive, cardioprotective, and possible antitumor attributes. The precise physiological role of resveratrol remains intangible, but it may participate in protecting plants from fungal infections and stress factors (1–4).

Resveratrol regulates multiple cellular and molecular events associated with tumor development. *In vivo*, resveratrol inhibits three major steps of carcinogenesis: initiation, promotion, and progression (5). It reduces the metabolism of arachidonate, which leads to the activation of carcinogenic compounds, and diminishes the transcription of cyclooxygenase-2, which catalyzes the formation of prostaglandins, anti-inflammatory factors that promote tumor growth. Resveratrol has antioxidant properties, which reduces the formation of reactive oxygen species that lead to genetic damage and successive tumor formation, and has antiproliferative effects (1–4, 6–9). Using relatively high (25–300 μM) concentrations, resveratrol has been shown to induce an artificial checkpoint at the G_1-S transition phase via induction of $p21^{WAF1}$ and down-modulation of G_1-S-specific cyclins/cyclin-dependent kinases, thus imposing G_1 arrest (10, 11). Aside from chemopreventive and cell cycle modulatory effects, resveratrol can induce apoptosis in certain tumor cells (12).

Most anticancer agents eradicate tumor cells by the induction of apoptosis (13). Tumor cells, in turn, have adopted various mechanisms to resist apoptosis. Natural inhibitors of apoptosis, such as Bcl-2 and IAP family

Received 6/2/03; revised 9/8/03; accepted 10/16/03.

Grant support: Fellowship from the UCLA Jonsson Comprehensive Cancer Center (A. R. Jazirehi).

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.

Requests for Reprints: Benjamin Bonavida, Department of Microbiology, Immunology, and Molecular Genetics, School of Medicine, University of California at Los Angeles, 10833 Le Conte Avenue, A2-060 CHS, Los Angeles, CA 90095-1747. Phone: (310) 825-2233; Fax: (310) 206-3865. Email: bbonavida@mednet.ucla.edu

members, protect the tumor cells from the apoptotic effects of various antineoplastic agents (13) via different mechanisms. Chemotherapy resistance is further reinforced by the emergence of multidrug resistant phenotype following initial chemotherapy administration due to the action of membrane-bound drug efflux pump (14–16).

The multidrug resistant phenotype plays a role in the drug resistance of multiple myeloma (MM) and non-Hodgkin's lymphoma (NHL; Refs. 15, 16) Thus, nontoxic agents that interfere with the function of drug efflux pump or negatively modulate the expression of antiapoptotic Bcl-2 and IAP family members can be effectively used in combination with chemotherapy in the clinical treatment of drug-resistant tumors.

Despite numerous efforts, the clinical treatment of patients with NHL and MM with conventional treatment regimes remains unsuccessful (17–19). In an attempt to treat such patients, ''third-line'' chemotherapeutic drugs such as paclitaxel have been used with variable and often limited success (17–19). Strategies to increase the efficacy and reverse the intrinsic or acquired resistance to such agents are crucial in the treatment of patients with NHL and MM. There is accumulating evidence that paclitaxel, either alone or in combination with other drugs, can be used in the treatment of patients with NHL and MM (18, 19). We have recently reported that tumor cells resistant to chemotherapeutic drugs can be sensitized by the same or other chemotherapeutic drugs to apoptosis induced by drugs or other agents such as tumor necrosis factor-related apoptosis-inducing ligand (TRAIL; Refs. 20, 21). The sensitization was due to the modification of apoptotic gene products involved in resistance. Because resveratrol can signal for apoptosis in sensitive lines, we hypothesized that paclitaxel-resistant NHL and MM cells that are also resistant to resveratrol-induced cytotoxicity may be sensitized by resveratrol to paclitaxel-induced apoptosis. We further hypothesized that resveratrol exerts its effects by interfering with signal transduction pathway(s) resulting in selective down-regulation of antiapoptotic factors and chemosensitization.

The objective of the present study was to test the above hypotheses and investigate (1) whether subtoxic concentrations of resveratrol can be used as a sensitizing agent to increase the cytotoxic effect of subtoxic concentrations of paclitaxel in paclitaxel-resistant NHL and MM cells, (2) the apoptotic signaling pathway used by resveratrol and paclitaxel for the induction of apoptosis, and (3) the potential signal transduction pathway modulated by resveratrol responsible for sensitization.

Materials and Methods

Cell Lines

The human Burkitt's lymphoma B-cell lines, Ramos (22) and Raji, were purchased from the American Type Culture Collection (Bethesda, MD). The establishment and characterization of human MM cell lines, 8226/S and 8226/Dox40, were previously described (23). Both cell lines were generously provided by Dr. William Dalton (Tampa, FL). The tumor cell lines were maintained in sterile 75 cm^2 tissue culture flasks in RPMI 1640 (Life Technologies, Inc., Bethesda, MD) supplemented with 10% (v/v) heat-inactivated fetal bovine serum as described previously (20). The cell lines were maintained at a density of 0.5×10^6 cells/ml and were subcultured every 2 days.

For the generation of peripheral blood mononuclear cells (PBMCs), whole blood from healthy donors was collected into 35 ml syringes with 0.5 ml sterile heparin. PBMCs were isolated by density centrifugation over a Ficoll-Hypaque density gradient (LSM, Organon Teknika Corp., Durham, NC), washed thrice in sterile PBS, and immediately used in cytotoxicity assays or stimulated with the B-cell mitogen [lipopolysaccharide (LPS); 20 ng/ml] for 3 days.

Reagents

A stock solution of 0.5 M resveratrol was kept at −20°C and a dilution of 10 μM was prepared fresh in DMSO for each experiment. Paclitaxel was dissolved in DMSO to make a stock solution of 6 mg/ml and was kept at room temperature. For each experiment, paclitaxel was diluted by DMSO to obtain the indicated concentrations. The DMSO concentration did not exceed 0.1% in any experiment. DMSO and resveratrol were purchased from Sigma Chemical Co. (St. Louis, MO). Paclitaxel was obtained from Bristol-Myers (New York, NY). Mouse anti-Bcl-x_L monoclonal antibody (mAb) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-poly(adenosine diphosphate-ribose) polymerase (PARP), anti-Bid, and anti-caspases-9, -8, and -7 polyclonal antibodies and the mouse anti-phospho-extracellular signal-regulated kinase 1/2 (ERK1/2; Thr^{202}/Tyr^{204}) mAb were purchased from Cell Signaling (Beverly, MA). Mouse anti-myeloid cell differentiation factor-1 (Mcl-1), rabbit anti-procaspase-3, FITC-labeled anti-caspase-3 mAbs, and rabbit anti-Bax polyclonal antibodies were purchased from PharMingen (San Diego, CA). Rabbit anti-apoptosis protease activating factor-1 (Apaf-1) polyclonal antibody and mouse anti-actin mAb were purchased from Chemicon International, Inc. (Temecula, CA). Rabbit anti-cellular inhibitor of apoptosis protein (c-IAP)-1, c-IAP-2, and X-linked inhibitor of apoptosis protein (XIAP) polyclonal antibodies were purchased from Trevegen (Gaithersburg, MD). Rabbit anti-survivin polyclonal antibody was purchased from Proscience (Poway, CA). Mouse anti-cytochrome *c* mAb and rat anti-Smac/DIABLO polyclonal antibody were purchased from PharMingen and Alexis Biochemicals (San Diego, CA), respectively. Rabbit anti-ERK1/2 polyclonal antibody, MEK1/2-specific inhibitor PD098059 [2-(2′-amino-3′-methoxyphenyl)-oxanaphtalen-4-one; 24, 25], and mitogen-activated protein kinase (MAPK) substrate-4 [172-192; sequence: N:Ala-Asp-Pro-Asp-His-Asp-His-Thr-Gly-Phe-Leu-Thr-Glu-Tyr-Val-Ala-Thr-Arg-Trp-Arg-Arg:C] were obtained from Biosource International (Camarillo, CA). U0126 [1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)-butadiene; 26] was purchased from Promega (Madison, WI). The Bcl-x_L inhibitor, 2-methoxyantimycin-A3 (2MAM-A3; 27), was purchased from Biomol (Plymouth, PA).

Resveratrol Pretreatment

Tumor cell lines (1×10^6 cells/ml) were grown in complete medium in 10 cm^2 tissue culture dishes (Life Technologies) and were treated with 10 μM resveratrol for 24 h. Thereafter, the cells were washed, fresh medium was added, and cells (2×10^6) were seeded into six-well Falcon plates (Fisher Scientific, Pittsburgh, PA). Indicated concentrations of paclitaxel were then added, and the cells were incubated for an additional 16 h for maximal cytotoxicity. At the end of the incubation period, the cells were harvested and subjected to propidium iodide (PI) staining according to the specifications of the PI staining kit (Roche Diagnostics, Indianapolis, IN) and evaluated by flow cytometric analysis.

Flow Cytometric-Based Analysis of Cell Cycle Distribution and Assessment of Apoptosis

The percentage of apoptotic cells was determined by evaluating PI-stained preparations (28) of resveratrol-pretreated cells with various concentrations of paclitaxel as described previously (20, 29). Cellular debris was excluded from the analysis by raising the forward scatter threshold, and the DNA content of the intact nuclei was recorded on a logarithmic scale. A minimum of 6000 events was collected on each sample and acquired in listmode by a PC Pentium computer. The percentage of apoptotic cells is represented as the percentage of hypodiploid cells accumulated at the sub-G_0 phase of the cell cycle.

Cell Viability and Inhibition of Cell Proliferation

Tumor cells (2×10^6 cells/treatment) were cultured in complete medium (control) or complete medium supplemented with various concentrations of resveratrol (1, 10, 50, 100, and 200 μM) for 24 h and various concentrations of paclitaxel (0.01–100 nM) for 16 h. At the end of the incubation period, the cells were harvested and a small aliquot of the cell suspension was removed and mixed with an equal volume of the 0.4% trypan blue dye solution, and total cell recovery and cell viability was determined by enumeration of the cells using light microscopy. The total number of untreated (control) cells was considered as the baseline (100%) for the analysis of cytostasis.

Evaluation of Active Caspase-3 Levels by Flow Cytometric Analysis

To validate the PI staining technique for the measurement of apoptosis, levels of active caspase-3 were evaluated. Ramos cells were treated under the conditions explained for PI staining and cell cycle analysis. At the end of the incubation period, the cells were washed once with ice-cold 1× PBS/0.1% BSA and were resuspended in 100 μl ice-cold 1× PBS/0.1% BSA. Fifty microliters of cell suspension (containing 2×10^6 cells) were aliquoted to each sample and fixed with the perm/fix solution (PharMingen) for 20 min. Thereafter, the cells were washed twice with 1× perm/wash (PharMingen) solution and stained with the FITC-labeled anti-active caspase-3 antibody (1:1000 predetermined dilution) for 30 min (light protected). Thereafter, the samples were washed once with 1× perm/wash solution followed by flow cytometric analysis (Coulter Electronics, Miami, FL). As negative control, the cells were stained with isotype control (pure IgG1) under the same conditions described above.

Immunoblot Analysis to Detect Alterations in Protein Expression

Ramos cells ($\sim 10^7$ cells/treatment) were either grown in complete medium (control) or complete medium supplemented with resveratrol (10 μM, 24 h), paclitaxel (10 nM, 16 h), or resveratrol (10 μM, 24 h) + paclitaxel (10 nM, 16 h). The cells were then lysed at 4°C in radioimmunoprecipitation assay buffer [50 mM Tris-HCl (pH 7.4), 1% NP40, 0.25% sodium deoxycholate, 150 mM NaCl] supplemented with one tablet of protease inhibitor cocktail (Complete Mini; Roche). A DC protein assay kit (Bio-Rad, Hercules, CA) was used to determine protein concentration. An aliquot of total protein lysate was diluted in an equal volume of 2 × SDS sample buffer [6.2 mM Tris (pH 6.8), 2.3% SDS, 5% mercaptoethanol, 10% glycerol, 0.02% bromophenol blue], boiled for 10 min, and kept at −80°C. The cell lysates (40 μg) were then electrophoresed on 12% SDS-PAGE gels (Bio-Rad). Western blot analysis was carried out as described previously (20). Levels of β-actin were confirmed to ensure equal loading of the samples. After incubation with the respective primary antibody, the nitrocellulose membranes were washed twice with PBS/0.1% Tween 20 and incubated for 30 min with horseradish peroxidase-conjugated anti-mouse or antirabbit IgG antibody (New England Biolabs, Beverly, MA). After washing thrice with Tris-buffered saline/0.1% Tween 20, the membranes were developed with a Lumiglo Western blot detection kit (New England Biolabs) in a Kodak (Rochester, NY) M35AX-OMAT processor. The relative intensity of the bands was assessed by densitometric analysis of the digitized images performed on an iMac computer (Apple Computer Inc., Cupertino, CA) using the public domain NIH image program (developed at the U.S. National Institutes of Health and available at http://rsb.info.nih.gov/nih-image/). Hence, we determined the percentage of alterations in protein expression.

Isolation of Cytosolic Fraction and Determination of Cytochrome *c* and Smac/DIABLO Contents

Ramos cells were grown under the conditions explained for immunoblot. At the end of the incubation period, the cells were washed twice with 1 ml ice-cold 1× PBS/0.1% BSA and were resuspended in 2 volumes of homogenization buffer [20 mM HEPES (pH 7.4), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM DTT, one tablet of Complete Mini protease inhibitor cocktail in 250 mM sucrose medium]. The cells were incubated on ice for 30 min to allow the cells to swell and lyse. The cells were then disrupted by 40 strokes of a 1.5 ml size Dounce glass homogenizer using a loose pestle to prepare the cell homogenates. The homogenate was centrifuged at 2500 × *g* at 4°C for 5 min to remove nuclei and unbroken cells. Mitochondria were pelleted by spinning the homogenate at 16,000 × *g* at 4°C for 30 min. The postmitochondrial supernatants were removed and

successively filtered through 0.2 and 0.1 μm Ultrafree MC filters (Millipore, Bedford, MA) to obtain cytosolic fractions and were spun down at 16,000 × *g* at 4°C for 15 min (21). The protein concentration of the supernatant was determined by the DC assay (Bio-Rad) and was mixed by 2× Laemelli sample buffer and analyzed by SDS-PAGE for determination of cytochrome *c* and Smac/DIABLO contents in the cytosolic fraction.

Analysis of Mitochondrial Membrane Potential by 3,3′-Dihexyloxacarbocyanine Iodide Staining

To quantitate the mitochondrial membrane potential ($\Delta\psi_m$), Ramos cells were stained with 3,3′-dihexyloxacarbocyanine iodide [$DiOC_6(3)$]. Briefly, Ramos cells (2×10^6) were seeded in 12-well plates (Costar Corp., Cambridge, MA) and were grown in either complete medium (control) or complete medium supplemented with resveratrol (10 μM), paclitaxel (10 nM), or resveratrol + paclitaxel (10 μM resveratrol, 24 h + 10 nM paclitaxel, 16 h). After incubation, 50 μl of 40 nM $DiOC_6(3)$ (Molecular Probes, Eugene, OR), a mitochondria-specific dye used to detect membrane depolarization (30), were added to each well and allowed to incubate for 30 min at 37°C. Cells were then washed twice in 1× PBS/0.1% BSA. After washing, 500 μl of 1× PBS/0.1% BSA were added to each sample and the cells were analyzed by flow cytometry.

Electrophoretic Mobility Shift Assay

After treatment with resveratrol and inhibitors, cells were pelleted and washed twice with ice-cold PBS. After washing, cells were lysed in 1.0 ml NP40 lysis buffer [10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP40] on ice for 5 min. Samples were centrifuged at 2000 × *g* at 4°C for 5 min in a microcentrifuge tube to pellet the nuclei and supernatants were subsequently removed. Nuclei were washed once in NP40 buffer and twice in ice-cold PBS. Nuclei were then lysed in nuclear extraction buffer [20 mM HEPES (pH 7.9), 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM DTT] and were sonicated at 4°C for 30 s. A DC protein assay kit (Bio-Rad) was used to determine the protein concentration of the nuclear extracts. Nuclear proteins were mixed for 30 min at room temperature with biotin-labeled oligonucleotide activator protein-1 (AP-1) probe (5′-CGCTTGATGACTCAGCCGGAA-3′) using the electrophoretic mobility shift assay (EMSA) kit purchased from Panomics, Inc. (Redwood City, CA) according to the manufacturer's instructions. Ten micrograms of nuclear extracts were subjected to denaturing 5% PAGE for 90 min in Tris-borate EDTA buffer (Bio-Rad) and transferred to Hybond-N^+ nylon transfer membrane using the Trans-Blot SD semidry transfer cell System (Bio-Rad). The membranes were cross-linked using UV Cross-linker FB-UVXL-1000 (Fisher Scientific, Pittsburg, PA) for 3 min and were exposed to Hyperfilm enhanced chemiluminescence films as described elsewhere.[2]

[2] M. I. Vega *et al.* Rituximab inhibits p38 MAPK activity in 2F7 B NHL and decreases IL-10 transcription: pivotal role of p38 MAPK in drug resistance, submitted for publication.

Reverse Transcriptase-PCR

Reverse transcription-PCR was used to detect transcriptional regulation of Bcl-x_L. Total RNA was extracted from ~10^7 cells for each different condition using the single-step guanidinium thiocyanate-chloroform method with 1.0 ml/sample of STAT-60 reagent (Tel-Test "B" Inc., Friendswood, TX). Three micrograms of total RNA were reversed to first-strand cDNA for 1 h at 42°C with Moloney murine leukemia virus reverse transcriptase (200 units; Life Technologies). The reverse transcriptase-PCR reaction mixture contained 20 μM random hexamer primers, 10 μM DTT, 125 μM each deoxynucleotide triphosphate, and 4 μl of 5× first-strand buffer (Life Technologies). Amplification of 2.5 μl of these cDNA by PCR was performed using the following Bcl-x_L gene-specific primers: forward 5′-ACCATGTCTCAGAGCAACCGGGAGCT-3′ and reverse 5′-TCATTTCCGACTGAAGAGTGAGCC-3′. Internal control for equal cDNA loading in each reaction was assessed using the following gene-specific glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers: GAPDH forward 5′-GAACATCATCCCTGCCTCTACTG-3′ and GAPDH reverse 5′-GTTGCTGTAGCCAAATTCGTTG-3′. The PCR amplifications of each specific DNA sequence were carried out using the Hot Start/Ampliwax method (31) using Platinum Tag (Life Technologies). Amplifications were performed with a DNA Thermo Cycler 480 (Perkin-Elmer, Norwalk, CT) with the following temperature cycling parameters: 95°C, 1 min; 60°C, 1 min; 35 cycles. The amplified products were analyzed on 2% agarose (Sigma Chemical) gels in Tris-borate EDTA [89 mM Tris base, 89 mM boric acid, 2 mM EDTA (pH 8.0)] and their relative concentrations were assessed by densitometric analysis of the digitized ethidium bromide-stained image using the public domain NIH image program.

Isobolographic Analysis for Determination of Synergy

Determination of the synergistic *versus* additive *versus* antagonistic cytotoxic effects of the combination treatment of the Ramos cell line by resveratrol and paclitaxel was assessed by isobolographic analysis as described previously (32). Briefly, isobolograms were constructed from a battery of combinations of various concentrations of resveratrol (1, 10, 20, 50, 75, 100, and 200 μM) with paclitaxel (0.1–1000 nM). Combinations yielding 35 ± 5% cytotoxicity were plotted as percentage of single agent alone that resulted in the same percentage of cytotoxicity (fractional inhibitory concentration: concentration of each agent in combination/concentration of each agent alone). When the sum of this fraction (fractional inhibitory concentration) is 1, the combination is additive and the graph is geometrically expressed as a straight line, when the sum is <1, the combination is synergistic and the graph appears as concave shape, and when the sum is >1, the combination is antagonistic and the graph is geometrically represented as convex shape.

Statistical Analysis

Assays were set up in triplicates and the results were expressed as means ± SD. Statistical analysis and *P*-value

determinations were done by two-tailed paired *t* test with a confidence interval of 95% for determination of the significance of differences between the treatment groups. $P < 0.05$ was considered to be significant. ANOVA was used to test for the significance among the groups. The InStat 2.01 software was used for the statistical analysis.

Results

Inhibition of Cell Recovery by Resveratrol and Paclitaxel on Ramos Cells

We have used the Ramos NHL B-cell line as representative of various cell lines for detailed investigation. Ramos cells were grown in complete medium in the absence (control) or presence of various concentrations (1, 10, 50, 100, and 200 μM) of resveratrol for 24 h. Viable cell recovery was determined microscopically by trypan blue dye exclusion assay (Fig. 1A). Untreated cells (control) were considered as the baseline (100%) for the analysis. Resveratrol inhibited cell recovery of the Ramos cell line and the inhibition was a function of the concentration used and was evident at a concentration as low as 10 μM (26.5 ± 4.7%). Maximum inhibition (73 ± 8.5%) was observed at 200 μM resveratrol (Fig. 1). Paclitaxel inhibited cell recovery of Ramos cells at concentrations of 10 and 100 nM (Fig. 1B). These results demonstrate that resveratrol (concentrations ≥ 10 μM) and paclitaxel (concentrations ≥ 10 nM) negatively modulate tumor cell growth in Ramos NHL B-cells.

Alterations in Cell Cycle Profile by Resveratrol and Paclitaxel

On the basis of the above data, subtoxic concentrations of resveratrol (10 μM) and paclitaxel (10 nM) were chosen for further analysis. Ramos cells were either grown in complete medium (control) or grown in complete medium supplemented with resveratrol (10 μM, 24 h), paclitaxel (10 nM, 16 h), or resveratrol (10 μM, 24 h) + paclitaxel (10 nM, 16 h). PI-stained preparations were subjected to DNA profile analysis by flow cytometry. Both resveratrol and paclitaxel arrested the Ramos cells at the G_2-M phase of the cell cycle. However, the combination of resveratrol and paclitaxel significantly reduced the rate of the G_2-M-arrested cells (Fig. 2; Table 1).

Resveratrol- and Paclitaxel-Induced Apoptosis in Ramos Cells

Ramos cells were treated under the same conditions used above for the analysis of cytostasis. After the incubation period, the cells were subjected to PI staining and flow cytometric analysis for assessment of apoptosis. The induction of apoptosis by resveratrol was initiated at 10 μM (13.8 ± 2.0%). The highest degree of apoptosis (36.1 ± 3.9%) was observed at 100 μM resveratrol and reached a plateau at 200 μM (Fig. 1C). Concentrations > 200 μM resveratrol were extremely toxic to the cells. Paclitaxel did not induce significant apoptosis at <10 nM but induced modest apoptosis at 100 nM (Fig. 1D).

Sensitization of the Ramos NHL B-Cell Line to Paclitaxel-Induced Apoptosis by Resveratrol

On the basis of the above findings, we examined whether resveratrol (10 μM) can sensitize Ramos cells to

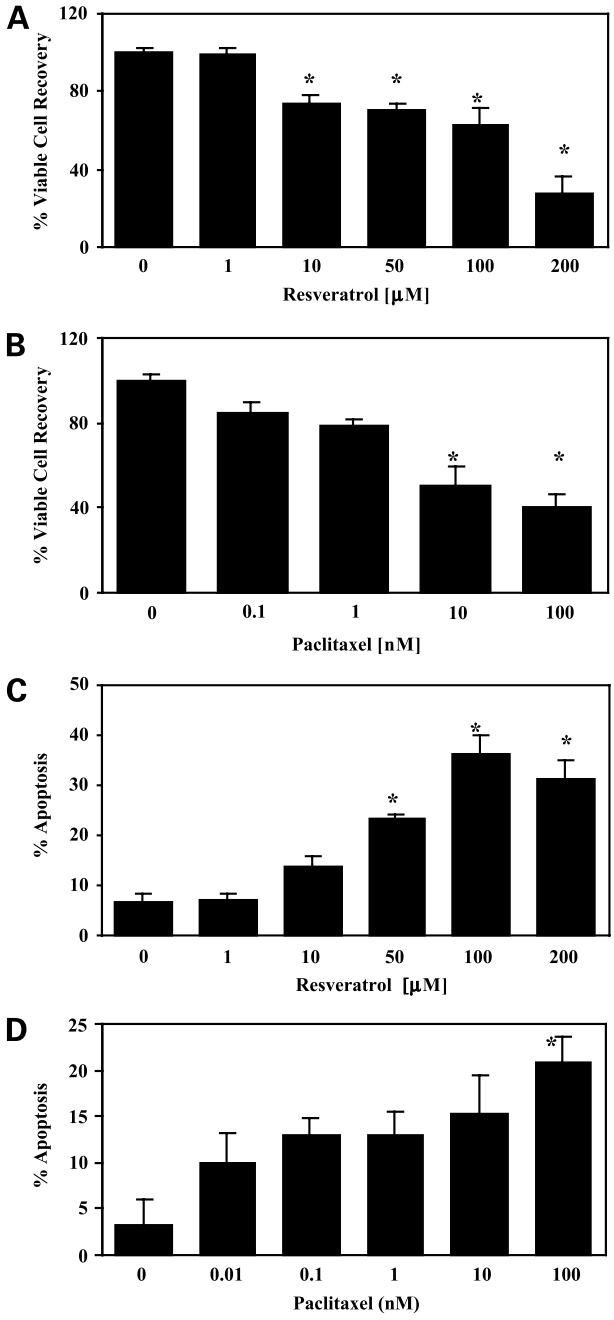

Figure 1. Resveratrol- and paclitaxel-induced cytostasis and apoptosis of Ramos NHL B-cells. Ramos cells were either left untreated or treated with various concentrations (1, 10, 50, 100, and 200 μM) of resveratrol for 24 h and various concentrations of paclitaxel (0.1 – 100 nM, 16 h). Thereafter, the cells were stained with PI and apoptosis was assessed by DNA fragmentation assay using flow cytometry. The percentage of apoptotic cells is represented as the percentage of the cells accumulated at sub-G_0 fraction of the cell cycle. For the evaluation of inhibition of cell proliferation, an aliquot of each of the above cell suspensions was mixed with an equal volume of the trypan blue dye and the total number of viable cells recovered was determined using light microscopy. Untreated (control) cells were considered as 100%. The samples were set up in triplicates. *Columns,* means of two independent experiments; *bars,* SD. *, $P < 0.05$, significant compared with untreated cells. **A** and **B,** percent viable cell recovery. **C** and **D,** percent apoptosis induced by resveratrol and paclitaxel, respectively.

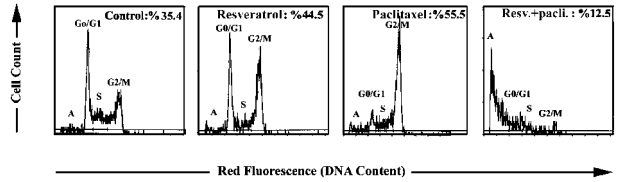

Figure 2. Cell cycle distribution and DNA profile analysis of resveratrol- and paclitaxel-treated Ramos cells. Ramos cells (1×10^6 cells/ml) were either left untreated or treated with resveratrol (10 μM, 24 h), paclitaxel (10 nM, 16 h), or resveratrol (10 μM, 24 h) + paclitaxel (10 nM, 16 h). At the end of the incubation period, the cells were divided into two equal aliquots. The first aliquot was stained with PI solution to assess both the cell cycle distribution and the DNA profile. *Upper right corner,* percentage of G_2-M-arrested cells. See also Table 1.

paclitaxel-induced apoptosis. Time kinetics studies (from 24 h to 5 days) demonstrated no significant difference in resveratrol-induced cytotoxicity in various time points. Thus, the shortest pretreatment time point (24 h) was used for further studies. Ramos cells were either grown in complete medium or pretreated with 10 μM resveratrol for 24 h. Thereafter, the cells were washed to remove excess resveratrol, fresh medium was added, and the cells were incubated with various concentrations of paclitaxel (0.1, 1, and 10 nM). At the end of the incubation period, the PI-stained preparations of cells were subjected to fluorescence-activated cell sorting analysis for the evaluation of DNA fragmentation. Resveratrol sensitized the Ramos cells to paclitaxel-induced apoptosis at concentrations $\geq$ 10 nM and 52.9 $\pm$ 3.2% (P = 0.01) of the cells underwent apoptosis with 10 nM paclitaxel (Fig. 3A). Isobolographic analysis shows that resveratrol potentiates paclitaxel-induced apoptosis in a synergistic manner (Fig. 3B). Time kinetics studies demonstrated that induction of apoptosis by the combination treatment started at 8–12 h post-treatment and reached the maximum levels by 16 h (Fig. 3C). Prolongation of the incubation period did not enhance the level of apoptosis.

The results of apoptosis achieved by DNA hypodiploidy (PI staining) were further confirmed by measuring the levels of activated caspase-3. Significant activation of caspase-3 was only observed in Ramos cells treated with combination of resveratrol and paclitaxel and was not detected by each agent used alone. Activation of caspase-3 paralleled the accumulation of the hypodiploid cells at the sub-G_0 region, which inversely correlated with the percentage of G_2-M-arrested cells (Table 1).

These results show that resveratrol decreases the threshold of apoptosis and renders paclitaxel-refractory Ramos cells sensitive to paclitaxel-mediated apoptosis in a synergistic manner.

Mechanism of Resveratrol-Mediated Sensitization of Ramos Cells to Paclitaxel-Induced Apoptosis

To assess the effects of resveratrol and paclitaxel, as single agents and in combination, on the integrity of mitochondria, Ramos cells were either left untreated or treated with resveratrol (10 μM, 24 h), paclitaxel (10 nM, 16 h), or resveratrol (10 μM, 24 h) + paclitaxel (10 nM, 16 h). Thereafter, the cells were stained with $DiOC_6(3)$, a mitochondrial specific dye, for 30 min at 37°C and analyzed by flow cytometry. Significant depolarization of mitochondria was observed by increased percentage of the cells with depolarized mitochondria (control, 10.4 $\pm$ 4.8; 10 μM resveratrol, 21.2 $\pm$ 0.1; 10 nM paclitaxel, 15.2 $\pm$ 3.3; 10 μM resveratrol + 10 nM paclitaxel, 81.3 $\pm$ 9.2) and by decrease in the mean fluorescence intensity when the Ramos cells were subjected to the combination treatment (Fig. 4).

Next, caspase activation and PARP cleavage were analyzed. Ramos cells were treated with resveratrol, paclitaxel, and combination. Thereafter, total cell lysates were subjected to immunoblotting. As single agents, neither resveratrol nor paclitaxel affected procaspases-8, -9, -3, and -7 and the DNA repair enzyme PARP. In contrast, the combination treatment resulted in significant down-regulation of procaspase-3 and activation of caspases-3 (Table 1), -9, and -7 and PARP cleavage (Fig. 5A) as indicated by the appearance of the cleaved fragments of the proteins. Caspase-8 was not completely processed (absence of the p18 active fragment) beyond the background levels by the combination treatment.

Preferential activation of caspase-9 (but not caspase-8) and collapse in $\Delta\psi_m$ suggest the involvement of type II mitochondrial apoptotic signaling pathway in resveratrol-mediated sensitization to paclitaxel. Thus, we examined the alterations in the expression level of individual gene products participating in the mitochondrial pathway. Ramos cells were treated under the abovementioned conditions and the levels of Smac/DIABLO and cytochrome *c*

Table 1. Cell cycle distribution and caspase-3 activation of resveratrol- and paclitaxel-treated Ramos cells

	G_0/G_1	S	G_2-M	Sub-G_0	Caspase-3
Control	32.6 $\pm$ 4.7	19.8 $\pm$ 3.2	38.4 $\pm$ 3.6	7.6 $\pm$ 4.2	7.2 $\pm$ 3.1
Resveratrol (10 μM)	29.0 $\pm$ 6.3	13.1 $\pm$ 2.8	46.4 $\pm$ 4.6	10.4 $\pm$ 3.4	6.9 $\pm$ 2.2
Paclitaxel (10 nM)	14.2 $\pm$ 2.9	15.3 $\pm$ 4.1	56.8 $\pm$ 3.1	14.6 $\pm$ 4.6	15.2 $\pm$ 3.8
Resveratrol + paclitaxel	13.6 $\pm$ 4.2	11.3 $\pm$ 3.6	14.3 $\pm$ 2.9	58.4 $\pm$ 3.8*	52.3 $\pm$ 4.6*

Note: The second aliquot of the samples in Fig. 2 was stained with FITC-labeled anti-active caspase-3 mAb for the measurement of the levels of active caspase-3. Both assays were analyzed by flow cytometry. Results are means $\pm$ SD of four independent experiments.
*$P < 0.05$, significant compared with paclitaxel treatment alone.

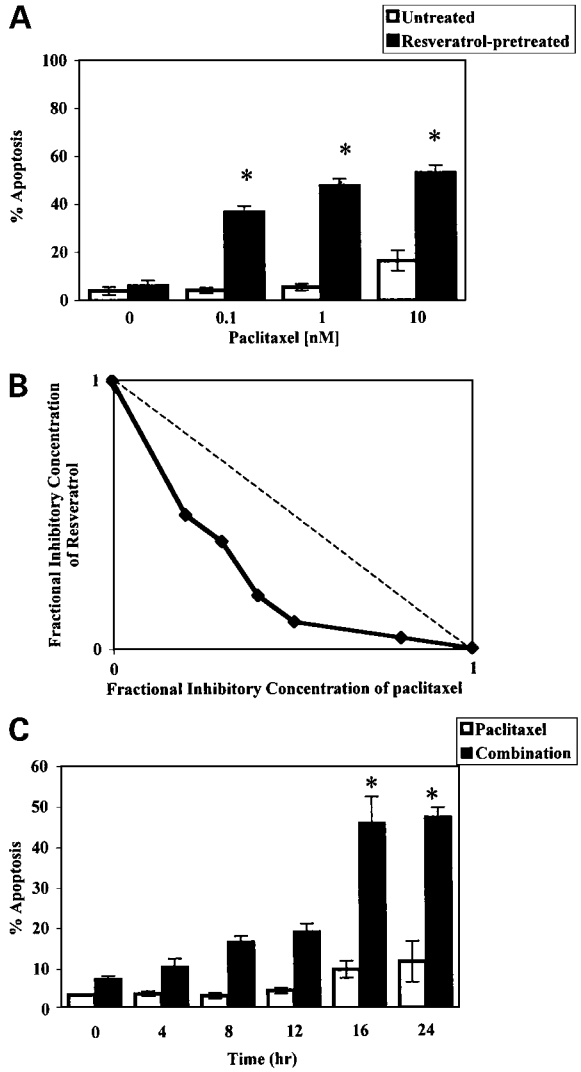

Figure 3. **A,** sensitization of Ramos cells to paclitaxel-induced apoptosis by resveratrol. **B,** isobolographic analysis for the determination of synergy (33). **C,** time kinetics analysis for the induction of synergy. Ramos cells were treated under the conditions explained in the legend of Fig. 2. Thereafter, the cells were harvested and stained with PI solution and apoptosis was assessed by DNA fragmentation using flow cytometry. The percentage of apoptotic cells is represented as the percentage of hypodiploid cells accumulated at the sub-G_0 phase of the cell cycle. The samples were set up in duplicates. *Columns,* means of two independent experiments; *bars,* SD. *, $P < 0.05$, significant compared with paclitaxel treatment alone.

were analyzed in the cytosolic fraction. Slight release of both apoptogenic molecules was observed by resveratrol and paclitaxel, which parallels their ability to induce marginal apoptosis. However, increased level of cytosolic Smac/DIABLO and cytochrome *c* was only observed by the combination treatment (Fig. 5B). Depletion of the mitochondrial cytochrome *c* and Smac/DIABLO paralleled their cytosolic accumulation that confirms their redistribution from mitochondria to the cytosol (data not shown).

Analysis of the expression of the proteins involved in the apoptotic pathway revealed that both resveratrol and paclitaxel modified several gene products that facilitated the apoptotic signaling pathway (Fig. 5C). Resveratrol inhibited the expression of Bcl-x_L (20%) and Mcl-1 (40%) and up-regulated the expression of the proapoptotic proteins Bax (240%) and Apaf-1 (386%). Likewise, paclitaxel inhibited the expression of the antiapoptotic proteins c-IAP-1 (70%), Bcl-x_L (37%), and Mcl-1 (49%) and up-regulated the expression of the proapoptotic proteins Bid (66%) and Apaf-1 (269%). Thus, both resveratrol and paclitaxel share common modulatory effects on gene expression profile such as Bcl-x_L, Mcl-1, and Apaf-1 and each has a specific effect on gene expression profile such as c-IAP-1 and Bid for paclitaxel and Bax for resveratrol.

Delineation of the molecular events on resveratrol and paclitaxel treatment of the Ramos cells demonstrates that each agent selectively alters the expression of particular proapoptotic and antiapoptotic components of the mitochondrial (type II) apoptotic signaling pathway (33). However, these changes were not sufficient for the activation of caspases and the full induction of apoptosis. The combination treatment, via functional complementation, resulted in tBid formation, cytosolic release of apoptogenic proteins, mitochondrial destabilization, significant down-regulation of some of the apoptosis regulatory proteins, processing of caspases, and cleavage of a death substrate, and apoptosis ensues.

Role of Bcl-x_L Down-Regulation by Resveratrol in the Sensitization of Ramos Cells to Paclitaxel-Induced Apoptosis

Previous reports suggest that Bcl-x_L expression is a resistant factor against apoptosis-inducing stimuli in lymphoma cell lines (29). We hypothesized that in Bcl-2-deficient Ramos cells (34) Bcl-x_L down-regulation by resveratrol significantly contributes to augmentation of paclitaxel-induced apoptosis. To confirm this hypothesis, 2MAM-A3, which specifically inhibits the function of Bcl-x_L (27), was used. The cells were grown in complete medium in the absence (control) or presence of 2MAM-A3 (20 μg/ml, 7 h) + paclitaxel (10 nM, 16 h). As shown, 2MAM-A3 alone was inefficient in killing Ramos cells (10.6±2.4%) but significantly augmented paclitaxel-induced apoptosis (37.8±1.2%; $P = 0.0093$; Fig. 6B).

The ERK1/2 MAPK is one of the main pathways frequently deregulated in tumor cells and contributes to the chemoresistance phenotype of tumor cells (35, 36). Further, the ERK1/2 pathway regulates Bcl-x_L expression via AP-1 transcription factor (37, 38). Thus, we examined alterations in the phosphorylation pattern of ERK1/2 on

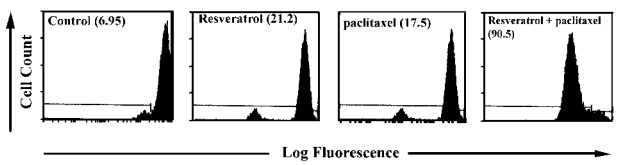

Figure 4. Representative of flow histograms demonstrating decrease in the mitochondrial transmembrane potential (ΔΨm) of the cells treated with resveratrol and paclitaxel.

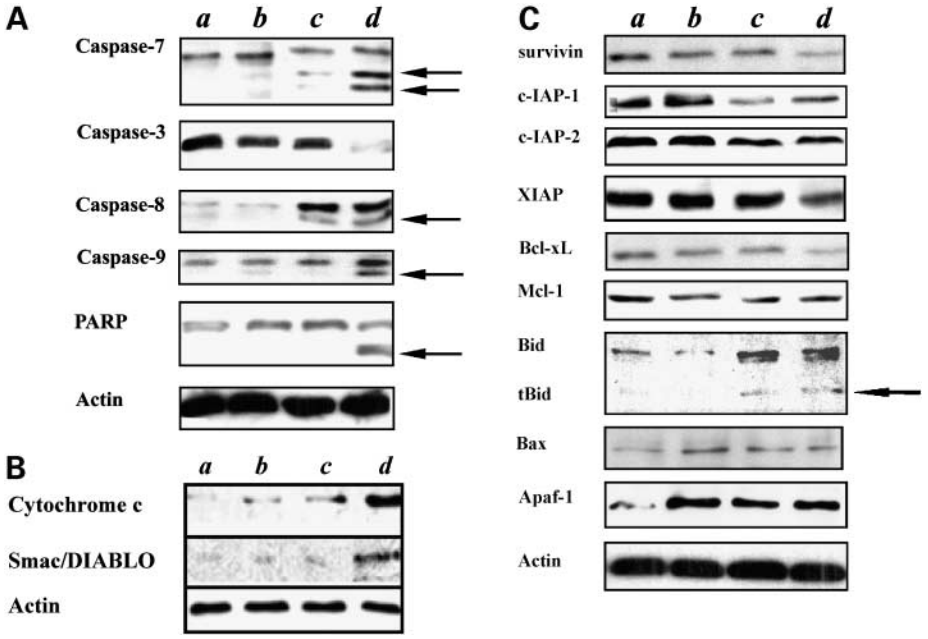

Figure 5. Immunoblot analysis for detection of proapoptotic and antiapoptotic proteins: **A,** activation of caspase cascade and PARP cleavage. **B,** determination of cytosolic levels of Smac/DIABLO and cytochrome *c*. **C,** modifications of apoptotic gene products by resveratrol, paclitaxel, and combination. Ramos cells were treated under the conditions explained in the legend of Fig. 2. Total cell lysates (40 μg) were subjected to immunoblotting. Results are representative of two independent experiments. (*a*) control, (*b*) resveratrol (10 μM), (*c*) paclitaxel (10 nM), and (*d*) resveratrol (10 μM) + paclitaxel (10 nM). *Arrows,* cleaved form of the apoptosis-related proteins.

resveratrol treatment. The cells were either left untreated or treated with resveratrol (10 μM, 6, 12, and 24 h) and total cell lysates were subjected to Western blot analysis using phosphospecific and non-phosphospecific anti-ERK1/2 antibodies. The results demonstrate that the ERK1/2 pathway is constitutively active in Ramos cells and resveratrol decreases the phosphorylation-dependent state of ERK1/2 in a time-dependent manner starting 12 h postresveratrol treatment while having no effect on the basal levels (phosphoindependent state) of ERK1/2 (Fig. 6A).

The involvement of the ERK1/2 MAPK signaling pathway in the resistance of Ramos cells to paclitaxel-induced apoptosis and its inhibition by resveratrol suggests that inhibition of this pathway should mimic resveratrol. Thus, we examined the effects of a series of inhibitors of the ERK1/2 MAPK pathway on paclitaxel-induced apoptosis. PD098059 and U0126 are noncompetitive specific inhibitors of ERK1/2 phosphorylation and activation (24–26) and MAPK substrate-4 inhibits phosphorylation of ERK1/2. Optimal concentrations of the inhibitors were determined by pilot studies, which are in accordance with previous reports (24–26). The cells were pretreated with the inhibitors (PD098059: 20 μg/ml, 45 min; U0126: 10 μM, 45 min; MAPK substrate-4: 15 μg/ml, 3 h) + paclitaxel (10 nM, 16 h) and subjected to PI staining (DNA fragmentation assay). Paclitaxel (10 nM) induced 12.9 ± 3.2% apoptosis. The inhibitors were nontoxic to the cells but sensitized the cells to paclitaxel-induced apoptosis. For instance, PD098059 at 20 μg/ml induced 10.9 ± 1.4% apoptosis, which was increased to 42.9 ± 5.8% ($P = 0.0235$) in the presence of 10 nM paclitaxel. U0126 (10 μM) induced 11.8 ± 2.9% apoptosis whereas combination with paclitaxel (10 nM)

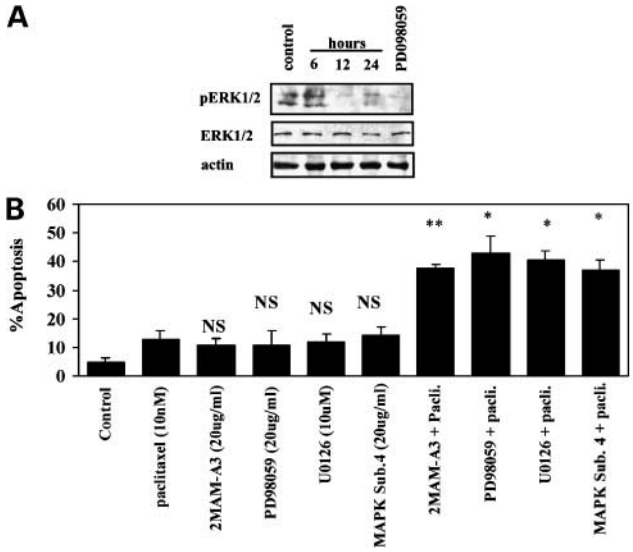

Figure 6. Signaling pathway involved in resveratrol-mediated Bcl-x_L down-regulation. **A,** inhibition of the ERK1/2 phosphorylation. Ramos cells were grown in complete medium in the absence (control) or presence of resveratrol (10 μM) for various time points (6, 12, and 24 h) or PD089059 (20 μg/ml, 45 min). Total cell lysates (40 μg) were subjected to Western blot analysis using phosphospecific and non-phosphospecific antibodies for the ERK1/2 MAPK. Results are representative of two independent experiments. **B,** sensitization of the Ramos cells to paclitaxel-induced apoptosis. Ramos cells were either left untreated (control) or pretreated with 2MAM-A3 (20 μg/ml, 7 h), U0126 (10 μM, 45 min), PD089059 (20 μg/ml, 45 min), or MAPK substrate-4 (15 μg/ml, 3 h). At the end of the indicated incubation periods, the cells were incubated with paclitaxel (10 nM, 16 h) and subjected to PI staining and flow cytometry. *Columns,* means of triplicate samples ($n = 2$); *bars,* SD. **, $P < 0.001$, very significant compared with paclitaxel treatment alone. *, $P < 0.01$, significant compared with paclitaxel treatment alone. *NS*, not significant compared with paclitaxel treatment alone.

resulted in 40.6 ± 3.1% (P = 0.0127) apoptosis. MAPK substrate-4 induced modest apoptosis (15 μg/ml: 14.3 ± 2.6%) while combination with paclitaxel resulted in 36.8 ± 3.8% (P = 0.0209) apoptosis in Ramos cells (Fig. 6B). These results show that pharmacological interruption of the ERK1/2 MAPK pathway, like resveratrol, sensitizes the Ramos cells to paclitaxel-induced apoptosis.

Resveratrol Decreases the DNA Binding Activity of AP-1 and AP-1-Dependent Bcl-x_L Gene Expression in Ramos Cells

Activation of the ERK1/2 MAPK pathway leads to the activation of AP-1 transcription factor and AP-1-dependent Bcl-x_L gene expression (37, 38). Because resveratrol decreased the activation of the ERK1/2 MAPK pathway, the alterations in the DNA binding activity of AP-1 on resveratrol treatment were examined. The cells were either left untreated (control) or treated with resveratrol (10 μM) for various time points (6, 12, and 24 h). Nuclear extracts were prepared and biotin-labeled oligonucleotides comprising the AP-1 binding site were used as probe in an EMSA assay; the AP-1 DNA binding activity was abolished in the presence of resveratrol. The ERK1/2 inhibitor PD098059 (20 μg/ml, 45 min) was used as positive control and diminished AP-1 DNA binding activity (Fig. 7A).

Visual inspection and computer database analysis (http://www.cbil.upenn.edu/cgi-bin/tess) revealed the presence of multiple AP-1 binding sites on Bcl-x_L promoter (data not shown). Because resveratrol decreased both AP-1 DNA binding activity and Bcl-x_L protein expression, we examined whether resveratrol modulates Bcl-x_L transcription. Ramos cells were either left untreated (control) or treated with resveratrol (10 μM), 2MAM-A3 (20 μg/ml), PD098059 (20 μg/ml), U0126 (10 μM), or MAPK substrate-4 (15 μg/ml) and total RNA was extracted and reverse transcribed to first-strand cDNA. Oligonucleotide primers derived from unique regions of Bcl-x_L mRNA were used in a PCR reaction. The 2MAM-A3 had no effect on Bcl-x_L gene expression while resveratrol and the inhibitors decreased Bcl-x_L transcription (Fig. 7B). Together, these results demonstrate the ability of resveratrol to decrease AP-1 DNA binding activity and AP-1-dependent Bcl-x_L transcription.

Resveratrol-Mediated Sensitization to Paclitaxel-Induced Apoptosis in Other B-Cell Lines but not in PBMCs

Resveratrol-mediated sensitization of Ramos cells to paclitaxel was examined in another NHL cell line. Resveratrol efficiently sensitized Raji cells to paclitaxel-induced apoptosis in a concentration-dependent manner (Table 2). Two MM cell lines (*i.e.*, 8226/S and 8226/Dox40) were also examined. Resveratrol significantly enhanced the killing of the 8226/S cells by paclitaxel at concentrations of 1 and 10 nM (Table 2). In contrast, resveratrol failed to sensitize the 8226/Dox40 cells to paclitaxel-induced apoptosis. Increased pretreatment period (up to 48 h) or longer incubation (up to 36 h) with a higher concentration (25 nM) of paclitaxel did not enhance the sensitivity of 8226/Dox40 cells (data not shown).

The cytotoxic effects of resveratrol and paclitaxel, alone and in combination, on freshly derived quiescent or mitogenically stimulated PBMCs were also examined. Freshly isolated PBMCs were grown in complete medium in the presence or absence of LPS (20 ng/ml, 3 days). At the end of the incubation period, the relative number of B-cells was determined by gating on the CD20-expressing population using fluorescence-activated cell sorting analysis, which revealed 3-fold increase in the number of B-cells (data not shown) on LPS stimulation. Thereafter, the cells were treated with resveratrol (10 μM, 24 h), paclitaxel (10 nM, 16 h), or combination as described above. As shown, both agents were nontoxic to PBMCs and resveratrol failed to sensitize PBMCs to paclitaxel (Table 2).

These results demonstrate that resveratrol-mediated sensitization to paclitaxel is not exclusive to Ramos cells. Noteworthy, the combination of resveratrol and paclitaxel induced minimal toxicity toward freshly derived quiescent or stimulated (rapidly proliferating) PBMCs.

Discussion

This study provides evidence for the first time that treatment with resveratrol can sensitize B-cell-derived tumor cells to the apoptotic effect of subtoxic concentrations (39) of paclitaxel. Resveratrol exerts its sensitizing effects by interruption with the cellular signaling pathways, induction of cell cycle arrest, and selective modification of apoptosis regulatory proteins. Paclitaxel also modified several apoptotic regulatory proteins. Whereas single agents resveratrol and paclitaxel were capable of modulating the expression of apoptotic

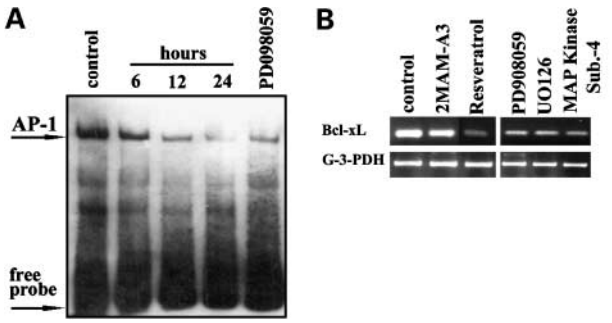

Figure 7. Resveratrol diminishes constitutive AP-1 DNA binding activity and AP-1-dependent Bcl-x_L gene expression. **A,** Ramos cells were grown in either complete medium (control) or complete medium supplemented with resveratrol (10 μM, 6, 12, and 24 h) or PD 089059 (20 μg/ml, 45 min). Ten micrograms of nuclear lysates were subjected to EMSA. Resveratrol (as early as 12 h) and PD089059 diminished constitutive DNA binding ability of AP-1. **B,** Ramos cells were either left untreated or treated with resveratrol (10 μM) or with the inhibitors [PD 089059 (20 μg/ml), U0126 (10 μM), and MAPK substrate-4 (15 μg/ml)] and total RNA was extracted and reverse transcribed to first-strand cDNA. cDNA of various samples (2.5 μg) was used in PCR analysis using Bcl-x_L primers. Resveratrol and the inhibitors down-regulate Bcl-x_L gene expression. Results are representative of two independent experiments.

Table 2. Resveratrol-mediated sensitization of NHL and MM cell lines but not PBMCs to paclitaxel-mediated apoptosis

	Control	Paclitaxel (nM)		
		0.1	1	10
Raji	9.51 ± 1.9	10.3 ± 2.9	11.3 ± 1.8	22.6 ± 1.1
Raji + resveratrol	9.83 ± 1.4	23.2 ± 2.6*	24.4 ± 3.9*	54.3 ± 1.6*
8226/S	4.7 ± 0.5	7.5 ± 0.1	32.8 ± 0.4	32.8 ± 0.1
8226/S + resveratrol	7.7 ± 0.7	14.6 ± 3.5^{NS}	43.8 ± 1.6*	57.8 ± 3.2*
8226/Dox40	4.4 ± 0.1	13.9 ± 0.5	8.5 ± 1.9	11 ± 2.4
8226/Dox40 + resveratrol	5.5 ± 0.4	6.8 ± 0.4^{NS}	8.3 ± 0.1^{NS}	10 ± 0.1^{NS}
PBMC	8.7 ± 1.3	ND	9.0 ± 1.6	9.6 ± 1.1
PBMC + resveratrol	8.4 ± 1.1	ND	10.6 ± 1.5^{NS}	9.8 ± 1.9^{NS}
PBMC (LPS)	10.2 ± 2.6	ND	9.6 ± 2.9	8.7 ± 2.2
PBMC (LPS) + resveratrol	11.0 ± 0.8	ND	17.7 ± 3.6^{NS}	14.4 ± 2.7^{NS}

Note: Freshly derived PBMCs were either left untreated or treated with LPS (20 ng/ml, 3 days). CD20-expressing PBMCs were at their log phase of growth and along with the tumor cell lines were treated under the conditions explained in the legend of Fig. 2. Thereafter, the cells were harvested, stained with the PI solution, and subjected to DNA fragmentation assay using flow cytometry. The percentage of apoptotic cells is represented as the percentage of hypodiploid cells accumulated at sub-G_0 fraction of the cell cycle. The samples were set up in duplicates. Results are represented as means ± SD of two independent experiments. NS, statistically not significant; ND, not determined.
*$P < 0.05$, significant compared with paclitaxel treatment alone.

regulatory gene products, these events were insufficient to induce apoptosis. Through functional complementation, however, the combination induced synergistic apoptosis. These findings suggest that resveratrol can overcome paclitaxel resistance in B-cell-derived tumor cell lines in an *in vitro* model system.

Inhibition of cell cycle progression is considered as a potential therapy for various cancers (40). Many anticancer agents disrupt the normal cell cycle dynamics, causing arrest in various phases of the cell cycle, which increases tumor cell's sensitivity to apoptosis-inducing agents (13). A low concentration of resveratrol (10 μM) and paclitaxel (10 nM) induced irreversible G_2-M arrest of NHL and MM cells as their removal from the culture medium failed to reestablish the normal cell cycle progression. The percentage of the G_2-M-arrested cells inversely correlated with the percentage of cells undergoing apoptosis (Fig. 2; Table 1). Possibly, the G_2-M-arrested cells are the primary targets of the combination treatment; however, apoptosis can also occur independent of the G_2-M-arrested cells. Resveratrol also caused G_2-M arrest in the Yoshida rat hepatoma model (41). Although resveratrol and paclitaxel both induced G_2-M arrest, their primary targets vary. Resveratrol inhibits the proliferation of the S-phase cells while paclitaxel blocks the progression of G_0/G_1 cells.

On the basis of our findings, we have schematically represented one potential mechanism used by resveratrol in sensitizing the Ramos cells to paclitaxel-induced apoptosis (Fig. 8). The regulatory effects of resveratrol on gene expression included down-regulation of Bcl-x_L and Mcl-1 antiapoptotic gene products along with significant up-regulation of Bax and Apaf-1 proapoptotic gene products. Paclitaxel caused G_2-M arrest and inhibited the expression of Bcl-x_L, Mcl-1, and c-IAP-1 while up-regulating Bid and Apaf-1 protein expression. The combination treatment, through complementation of these apoptotic regulatory proteins, resulted in the execution of the apoptotic signaling pathway. Both resveratrol and paclitaxel shared similar modifications of apoptosis regulatory proteins (Bcl-x_L, Mcl-1, and Apaf-1) and exhibited unique modifications (Bax for resveratrol and c-IAP-1 and Bid for paclitaxel).

The cytosolic adapter molecule Apaf-1 plays a critical role in the mitochondrial apoptotic pathway. To obtain its optimal conformation and enzymatic activity, caspase-9 requires Apaf-1 (42). Overexpression of Apaf-1 cleaved Bid and sensitized the HL-60 leukemia cells to paclitaxel and etoposide (43), which was inhibited by XIAP (30). Up-regulation of Apaf-1 by Adriamycin contributes to the enhanced sensitivity of MM cells to TRAIL (20). Further, our findings on the up-regulation of Apaf-1 by resveratrol were confirmed in a recent study (44).

Resveratrol up-regulated Bax and thereby facilitated mitochondrial activation. The proapoptotic Bcl-2 member Bax and the caspase-8 cleaved form of Bid, tBid, insert into the mitochondrial membrane and form oligomers large enough to permit the diffusion of apoptogenic proteins from the mitochondria to the cytosol (45, 46). Bax overexpression enhances the sensitivity of ovarian cell lines to paclitaxel-mediated apoptosis in *in vitro* and *in vivo* model systems (47, 48) and induces apoptosis in Burkitt's lymphoma cell lines (49). Additionally, induction of Bax decreases the threshold of tumors to paclitaxel-induced apoptosis (50, 51). Resveratrol has been shown to increase intracellular levels of Bax (52) and we concur with these findings.

The BH3 domain-only protein Bid induces a conformational change resulting in Bax activation prior to its insertion into the mitochondrial outer membrane (53). Overexpression of Bid increased the sensitivity of tumor cells to ribonucleotide reductase inhibitors (54). Thus, induction of Bid by paclitaxel contributes to the apoptotic signaling pathway.

Resveratrol and paclitaxel inhibited Bcl-x_L and Mcl-1 expression. These play an important role in inhibiting apoptosis. Resveratrol has been shown to up-regulate Bax and down-regulate Bcl-x_L expression concurrent with an increase in sub-G_1 fraction (55). Overexpression of Bcl-x_L inhibits paclitaxel-mediated apoptosis (56). Resveratrol- and paclitaxel-mediated modulation of Bcl-x_L by these investigators is in agreement with our findings. Thus, we considered that resveratrol-mediated down-regulation of Bcl-x_L expression may be responsible, in large part, for the observed sensitization to paclitaxel. This was confirmed by functional impairment of Bcl-x_L, which sensitized the cells to paclitaxel-induced apoptosis (Fig. 6B). It has been reported that the ERK1/2 MAPK pathway regulates Bcl-x_L expression via AP-1 (37, 38). Thus, we examined whether resveratrol interferes with this pathway. Indeed, resveratrol partially blocked the phosphorylation of ERK1/2 and diminished the DNA binding activity of AP-1 and AP-1-dependent Bcl-x_L gene expression (Figs. 6A and 7, A and B). The role of the ERK1/2 pathway in the regulation of Bcl-x_L expression was corroborated by using specific inhibitors of the ERK1/2 pathway, which decreased Bcl-x_L gene expression (Fig. 7B) and sensitized the cells to paclitaxel-induced apoptosis (Fig. 6B). Inhibition of the ERK1/2 pathway by resveratrol has been previously reported (57). A major survival role of high expression of Mcl-1 in myeloma (58) and lymphoma cell lines (59, 60) has been proposed. Down-regulation of Mcl-1 expression by antisense approach (61) or immunotherapy (anti-CD20 mAb; 62) sensitized myeloma and leukemia cells to chemotherapy. Decreased Mcl-1 protein stability paralleled the increased sensitivity of paclitaxel-resistant ovarian carcinoma cell lines to paclitaxel (63). In Bcl-2-deficient Ramos cells (34), high levels of Bcl-x_L and Mcl-1 may indirectly interfere with downstream events that lead to apoptosis, which in turn may contribute to drug resistance. In agreement with these reports, our results suggest that down-regulation of Bcl-x_L and Mcl-1 by both agents may be partly implicated in the enhanced sensitivity of Ramos cells to paclitaxel-induced apoptosis. Indeed, Mcl-1 expression is also regulated by the ERK1/2 pathway (64, 65). Thus, inhibition of the ERK1/2 signaling pathway and decreased expression of Bcl-x_L and Mcl-1 by resveratrol might, in part, be responsible for sensitization to paclitaxel. Our results agree with previous reports where inhibition of the ERK1/2 pathway augmented the cytotoxic effects of paclitaxel (66, 67).

Paclitaxel inhibited c-IAP-1 expression and thus potentiated the activation of caspases-9 and -3. IAP family members suppress apoptotic pathways initiated by stimuli that release cytochrome *c* from mitochondria via binding to and ablating of the activation of caspases-9, -3, -6, and -7 but not the initiator caspase-8 (68, 69). Thus, high levels of IAPs confer apoptosis resistance to the tumor cells. Likewise, high expression of c-IAP-1, c-IAP-2, and XIAP confers survival advantage and chemoresistance to tumor cells (69). Alternatively, decreased levels of IAPs can potentially activate multiple signaling pathways that result in cell cycle delay or block in the G_1-S or G_2-M transition phase. Because most chemotherapeutic agents use the mitochondrial pathway and the fact that IAPs do not bind to caspase-8, high expression of IAPs represents an additional level of protection of NHL and MM cells against chemotherapy (68, 69).

The present findings (cytosolic release of cytochrome *c* and Smac/DIABLO and activation of caspase-9) demonstrate that the combination treatment activates the mitochondrial pathway of apoptosis. Electron microscopy and tomography data demonstrate that mitochondrial intermembrane space is separated from the crystae lumen. About 85–97% of the cytochrome *c* reside in the intramitochondrial crystae (70, 71). For its redistribution and release from mitochondria, cytochrome *c* should first cross the crystae lumen/intermembrane space boundary and

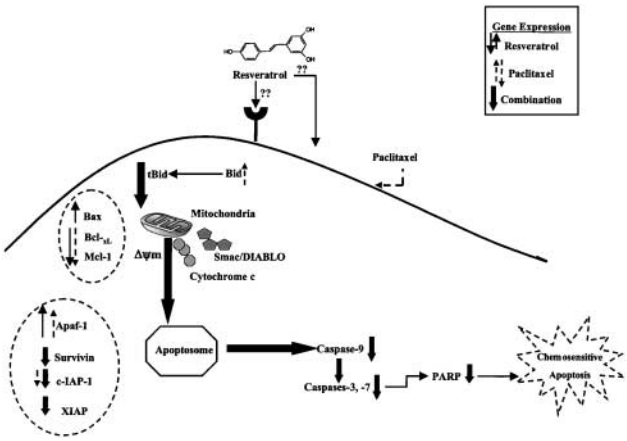

Figure 8. Proposed model of resveratrol-mediated sensitization of Ramos cells to paclitaxel-induced apoptosis. Resveratrol, either via a receptor-mediated pathway or by direct diffusion through the plasma membrane, interferes with apoptosis signaling pathways via regulation of gene expression. The molecular events triggered by resveratrol include decrease in the expression of Bcl-x_L (via inhibition of the ERK1/2 MAPK pathway and AP-1 transcriptional activity) and Mcl-1 antiapoptotic proteins plus significant induction of proapoptotic Bax and Apaf-1. Paclitaxel down-regulates antiapoptotic protein c-IAP-1 and up-regulates the expression of proapoptotic proteins Bid and Apaf-1. Yet, these various modulatory effects by themselves are inadequate for the full induction of apoptosis. The combination treatment, via functional complementation, results in the formation of proapoptotic tBid and the induction of apoptosis. tBid migrates to and resides in the mitochondrial outer membrane. Inherent Bcl-2 deficiency decreased levels of Mcl-1 and Bcl-x_L by resveratrol and paclitaxel that are more pronounced by the combination treatment, and the presence of tBid and high levels of Bax will alter the ratio of proapoptotic/antiapoptotic Bcl-2 family members. Decrease in these ratios, which are key determinants in the cellular fate in response to noxious stimuli, will result in the formation of permeability transition pore and collapse in $\Delta\psi_m$ and will facilitate the cytosolic release of apoptogenic molecules (cytochrome *c* and Smac/DIABLO). Smac/DIABLO binds to and neutralizes the inhibitory effects of IAPs. Hence, increased levels of Apaf-1 in combination with cytochrome *c* and dATP/ATP facilitates the assembly of the apoptosome complex. Through autocatalytic processing, caspase-9 becomes activated, and in the presence of diminished levels of natural inhibitors (survivin, XIAP, and c-IAP-1), it activates caspases-3 and -7 to subsequently cleave PARP and induce apoptosis. *Solid and dashed arrows,* signaling molecules altered by resveratrol and paclitaxel, respectively. *Large solid arrows,* modulation/activation by the combination.

then be released into the cytosol (72). Redistribution of mitochondrial apoptogenic proteins explains the absence of significant decrease in $\Delta\psi_m$ (Fig. 4), cytosolic Smac/DIABLO and cytochrome *c*, and apoptosis (Fig. 3) even when each agent has selectively altered the expression of apoptosis regulatory proteins (Fig. 5B). The formation of tBid, only observed by the combination treatment, might be the ultimate step in mitochondrial destabilization, which releases Smac/DIABLO and cytochrome *c* to the cytosol. Through physical interaction, Smac/DIABLO neutralizes the IAPs and cytochrome *c* participates in the formation of the apoptosome complex, both of which are crucial for caspase cascade activation and the full induction of apoptosis (73).

The execution phase of apoptosis occurs by the activation and function of caspases, which cleave key substrates that orchestrate the death process. Resveratrol and paclitaxel, in combination and not as single agents, via modulation of the expression of IAP and Bcl-2 family members in conjunction with Smac/DIABLO and cytochrome *c*, remove the inhibitory blocks and activate caspases (-9, -3, and -7), cleave PARP and apoptosis ensues (Fig. 5A)

Noteworthy, resveratrol-mediated enhanced sensitivity to paclitaxel was not limited to Ramos and was applicable to other NHL and MM cell lines (Table 2). While the study focused on paclitaxel for signaling, *cis*-diamine dichloride platinum and other apoptosis-inducing agents in combination with resveratrol were as efficient as paclitaxel to induce apoptosis in NHL and MM cell lines (data not shown). Interestingly, resveratrol was incapable of sensitizing the 8226/Dox40 cells to paclitaxel-induced apoptosis. These cells were not inherently resistant to the sensitizing effects of resveratrol, as they were efficiently sensitized to TRAIL and *cis*-diamine dichloride platinum (data not shown). Furthermore, subtoxic concentrations of Adriamycin rendered the 8226/Dox40 cells sensitive to TRAIL-mediated apoptosis (20).

Resveratrol did not induce apoptosis in freshly derived quiescent or mitogenically stimulated (rapidly proliferating) human PBMCs and the combination of resveratrol and paclitaxel did not enhance the sensitivity of PBMCs beyond the background levels (Table 2). These results denote the ability of resveratrol to improve the sensitivity of drug-refractory NHL and MM cell lines to paclitaxel-mediated apoptosis while having negligible cytotoxicity against the normal cells.

Evidence suggests a beneficial role for paclitaxel, either alone or combined with other drugs, in the treatment of patients with MM or NHL (18, 19). Our results demonstrate for the first time that resveratrol via selective modification of expression of apoptotic regulatory proteins and induction of cell cycle arrest can reverse the chemoresistance phenotype of B-cell-derived tumor cell lines in an *in vitro* model system and potentiate the apoptotic effects of apoptosis-inducing stimuli. Validation of our *in vitro* findings with an *in vivo* model system is warranted for their potential clinical application in the management of B-cell malignancies.

Acknowledgments

We thank Dr. William Dalton (Tampa, FL) for providing the multiple myeloma cell lines, Drs. XiaoHu Gan and Sara Huerta for assistance in the analysis of active caspase-3 by flow cytometry, Dr. Mario Vega for assistance with the EMSA assay, and Kate Dinh for assistance in the preparation of manuscript.

References

1. Bhat KPL, Kosmeder JW, Pezzuto JM. Biological effects of resveratrol. Antioxid Redox Signal. 2001;3:1041 – 64.

2. Cal C, Garban H, Jazirehi A, Yeh C, Mizutani Y, Bonavida B. Resveratrol and cancer: chemoprevention, apoptosis, and chemoimmuno-sensitizing activities. Curr Med Chem Anti-Cancer Agents. 2003;3:77 – 93.

3. Bhat KPL, Pezzuto JM. Cancer chemopreventive activity of resveratrol. Ann NY Acad Sci. 2002;957:210 – 29.

4. Roemer K, Mahyar-Roemer M. The basis for the chemopreventive action of resveratrol. Drugs Today. 2002;38:571 – 80.

5. Jang M, Cai L, Udeani GO, Slowing KV, Thomas CF, Beecher CW, et al. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. Science. 1997;275:218 – 20.

6. Subbaramaiah K, Chung WJ, Michaluart P, Telang N, Tanabe T, Inoue H, et al. Resveratrol inhibits cyclooxygenase-2 transcription and activity in phorbol ester-treated human mammary epithelial cells. J Biol Chem. 1998;273:21875 – 82.

7. Zoberi I, Bradbury CM, Curry HA, Bisht KS, Goswami PC, Roti JL, et al. Radiosensitizing and anti-proliferative effects of resveratrol in two human cervical tumor cell lines. Cancer Lett. 2002;175:165 – 73.

8. Moreno JJ. Resevratrol modulates arachidonic acid release, prostaglandin synthesis, and 3T6 fibroblast growth. J Pharmacol Exp Ther. Conference. 2000;294:333 – 8.

9. Martinez J. Moreno JJ. Effect of Resveratrol, a natural polyphenolic compound, on reactive oxygen species and prostaglandin production. Biochem Pharmacol. 2000;59:865 – 70.

10. Ahmad N, Adhami VM, Afaq F, Feyes DK, 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. 2001;7:1466 – 73.

11. Joe AK, Liu H, Suzui M, Vural ME, Xiao D, Weinstein IB. Resveratrol induces growth inhibition, S-phase arrest, apoptosis, and changes in biomarker expression in several human cancer cell lines. Clin Cancer Res. 2002;8:893 – 903.

12. Dörrie J, Gerauer H, Wachter Y, Zunino SJ. Resveratrol induces extensive apoptosis by depolarizing mitochondrial membranes and activating caspase-9 in acute lymphoblastic leukemia cells. Cancer Res. 2001;61:4731 – 9.

13. Ferreria CG, Epping M, Kruyt FAE, Giaccone G. Apoptosis: target of cancer therapy. Clin Cancer Res. 2002;8:2024 – 34.

14. Bebawy M, Morris MB, Roufogalis BD. Selective modulation of P-glycoprotein-mediated drug resistance. Br J Cancer. 2001;85:1998 – 2003.

15. Nooter K, Stoter G. Molecular mechanisms of multidrug resistance in cancer chemotherapy. Pathol Res Pract. 1996;192:768 – 80.

16. Sandor V, Wilson W, Fojo T, Bates SE. The role of MDR-1 in refractory lymphoma. Leuk Lymphoma. 1997;28:23 – 31.

17. Emmanouilides C, Jazirehi A, Bonavida B. Rituximab-mediated sensitization of non-Hodgkin's lymphoma (NHL) B-cells to cytotoxicity induced by paclitaxel, gemcitabine and vinorelbine. Cancer Biother Radiopharm. 2002;17:621 – 30.

18. Goss P, Stewart AK, Couture F, Klasa R, Gluck S, Kaizer L, et al. Combined results of two phase II studies of taxol (paclitaxel) in patients with relapsed or refractory lymphomas. Leuk Lymphoma. 1999;34:295 – 304.

19. Miller HJ, Leong T, Khandekar JD, Greipp PR, Gertz MA, Kyle RA. Paclitaxel as the initial treatment of multiple myeloma: an Eastern Cooperative Oncology Group Study. Am J Clin Oncol. 1998;21:553 – 6.

20. Jazirehi AR, Ng C-P, Gan X-H, Schiller G, Bonavida B. Adriamycin sensitized the Adriamycin-resistant 8226/Dox40 human multiple myeloma cells to Apo2L/TRAIL-mediated apoptosis. Clin Cancer Res. 2001;7:3874 – 83.

21. Ng C-P, Bonavida B. A new challenge for successful immunotherapy

by tumors that are resistant to apoptosis: two complementary signals to overcome cross-resistance. Adv Cancer Res. 2002;85:145 – 74.

22. Klein G, Giovanella B, Westman A, Stehlin JS, Mumford D. An EBV-genome-negative cell line established from an American Burkitt's lymphoma; receptor characteristics. EBV infectibility and permanent conversion into EBV-positive sublines by *in vitro* infection. Intervirology. 1975;5:319 – 34.

23. Dalton WS, Durie BG, Alberts DS, Gerlach JH, Cress AE. Characterization of a new drug-resistant human myeloma cell line that express P-glycoprotein. Cancer Res. 1986;46:5125 – 30.

24. Dudley DT, Pang L, Decker SJ, Bridges AJ, Saltiel AR. A synthetic inhibitor of the mitogen-activated protein kinase cascade. Proc Natl Acad Sci USA. 1995;92:7686 – 9.

25. Alessi DR, Cuenda A, Cohen P, Dudley DT, Saltiel AR. PD098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase *in vitro* and *in vivo*. J Biol Chem. 1995;270:27489 – 94.

26. Favata MF, Horiuchi KY, Manos EJ, Daulerio AJ, Stradley DA, Feeser WS, et al. Identification of a novel inhibitor of mitogen-activated protein kinase kinase. J Biol Chem. 1998;273:18623 – 32.

27. Tzung S-P, Kim CM, Basanez G, Giedt CD, Simon J, Zimmerberg J, et al. Antimycin A mimics a cell death-inducing Bcl-2 homology domain 3. Nat Cell Biol. 2001;3:183 – 91.

28. Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J Immunol Methods. 1991;139:271.

29. Jazirehi AR, Gan X-H, De Vos S, Emmanouilides C, Bonavida B. Rituximab (anti-CD20) selectively modifies Bcl-x_L and Apaf-1 expression and sensitizes human non-Hodgkin's lymphoma B cell lines to paclitaxel-induced apoptosis. Mol Cancer Ther. 2003;2:1183 – 93.

30. Perkins CL, Fang G, Kim CN, Bhalla KN. The role of Apaf-1, caspase-9, and Bid proteins in etoposide- or paclitaxel-induced mitochondrial events during apoptosis. Cancer Res. 2000;60:1645 – 53.

31. Birch DE. Simplified hot start PCR. Nature (Lond). 1996;381:445 – 6.

32. Berenbaum MC. A method for testing for synergy with any number of agents. J Infect Dis. 1978;137:122.

33. Scaffidi C, Fluda S, Srinivasan A, Friesen C, Li F, Tomaselli KJ, et al. Two CD95 (APO-1/Fas) signaling pathways. EMBO J. 1998;17: 1675 – 87.

34. Shan D, Ledbetter JA, Press OW. Apoptosis of malignant human B cells by ligation of CD20 with mAbs. Blood. 1998;91:1644 – 52.

35. Lee JT Jr., McCubrey JA. The Raf/MEK/ERK signal transduction cascade as a target for chemotherapeutic intervention in leukemia. Leukemia. 2002;16:486 – 507.

36. Fan M, Chambers TC. Role of mitogen-activated protein kinases in the response of tumor cells to chemotherapy. Drug Resist Updates. 2001;5:253 – 67.

37. Karin M. The regulation of AP-1 activity by mitogen-activated protein kinases. J Biol Chem. 1995;270(28):16483 – 6.

38. Sevilla L, Zaldumbide A, Pognonec P, Boulukos KE. Transcriptional regulation of the bcl-x gene encoding the anti-apoptotic Bcl-x_L protein by Ets, Rel/NF-κB, STAT and AP-1 transcription factor families. Histol Histopathol. 2001;16:595 – 601.

39. Rowinsky EK, Donehower RC. Antimicrotubule agents. Cancer chemo-therapy and biotherapy. 2nd ed. Chabner BA, Longo DL, editors. Philadelphia: Lippincott-Raven Publishers; 1996. p. 263 – 90.

40. Collins K, Jacks T, Paveltich NP. The cell cycle and cancer. Proc Natl Acad Sci USA. 1997;94:2776 – 8.

41. Carbo N, Costelli P, Baccino FM, Lopez-Soriano FJ, Argiles JM. Resveratrol, a natural product present in wine, decreases tumor growth in a rat tumor model. Biochem Biophys Res Commun. 1999;254: 739 – 43.

42. Cain K, Bratton SB, Cohen GM. The Apaf-1 apoptosome: a large caspase-activating complex. Biochemie. 2002;84:203 – 14.

43. Perkins C, Kim CN, Fang G, Bhalla KN. Overexpression of Apaf-1 promotes apoptosis of untreated and paclitaxel- or etoposide-treated HL-60 cells. Cancer Res. 1998;58:4561 – 6.

44. Narayanan BA, Narayanan NK, Stoner GD, Bullock BP. Interactive gene expression pattern in prostate cancer cells exposed to phenolic antioxidants. Life Sci. 2002;70:1821 – 39.

45. Kudla G, Montessuit S, Eskes R, Berrier C, Martinou JC, Ghazi A, et al. The destabilization of lipid membranes induced by the C-terminal fragment of caspase-8 cleaved Bid is inhibited by the N-terminal fragment. J Biol Chem. 2000;275:22713 – 8.

46. Korsmeyer SJ, Wei MC, Saito M, Weiler S, Oh KJ, Schlesinger PH. Pro-apoptotic cascade activates BID, which oligomerizes BAK or BAX into pores that result in the release of cytochrome *c*. Cell Death Differ. 2000; 7:1166 – 73.

47. De Feudis P, Vignati S, Rossi C, Mincioni T, Giavazzi R, D'Incalci M, et al. Driving p53 response to Bax activation greatly enhances sensitivity to taxol by inducing massive apoptosis. Neoplasia. 2000;2: 202 – 7.

48. Tsuruta Y, Mandai M, Konishi I, Kuroda H, Kusakari T, Yura Y, et al. Combination effect of adenovirus-mediated pro-apoptotic bax gene transfer with cisplatin or paclitaxel treatment in ovarian cancer cell lines. Eur J Cancer. 2001;37:531 – 41.

49. Szocinski JL, Khaled AR, Hixon J, Halverson D, Funakoshi S, Fanslow WC, et al. Activation-induced cell death of aggressive histology lymphomas by CD40 stimulation: induction of bax. Blood. 2002;100: 217 – 23.

50. Strobel T, Swanson L, Korsmeyer S, Cannistra SA. BAX enhances paclitaxel-induced apoptosis through a p53-independent pathway. Proc Natl Acad Sci USA. 1996;93:14094 – 9.

51. Strobel T, Kraeft SK, Chen LB, Cannistra SA. BAX expression is associated with enhanced intracellular accumulation of paclitaxel: a novel role for BAX during chemotherapy-induced cell death. Cancer Res. 1998;58:4776 – 81.

52. Mahyar-Roemer M, Katsen A, Mestres P, Roemer K. Resveratrol induces tumor cell apoptosis independently of p53 and precedes by epithelial differentiation, mitochondrial proliferation and membrane potential collapse. Int J Cancer. 2001;94:615 – 22.

53. Roucou X, Rostovtseva T, Montessuit S, Martinou JC, Antonsson B. Bid induces cytochrome *c*-impermeable Bax channels in liposomes. Biochem J. 2002;363:547 – 52.

54. Krajewska M, Zapata JM, Meinhold-Heerlein I, Hedayat H, Monks A. Expression of Bcl-2 family member Bid in normal and malignant tissues. Neoplasia. 2002;4:129 – 40.

55. Nakagawa H, Kiyozuka Y, Uemura Y, Shikata N, Hioki K, Tsubura A. Resveratrol inhibits human breast cancer cell growth and may mitigate the effect of linoleic avid, a potent breast cancer cell stimulator.J Cancer Res Clin Oncol. 2001;127:258 – 64.

56. Ibrado AM, Liu L, Bhalla K. Bcl-x_L overexpression inhibits progression of molecular events leading to paclitaxel-induced apoptosis of acute myeloid leukemia HL-60 cells. Cancer Res. 1997;57:1109 – 16.

57. El-Mowafy AM, White RE. Resveratrol inhibits MAPK activity and nuclear translocation in coronary artery smooth muscle: reversal of endothelin-1 stimulatory effects. FEBS Lett. 1999;451:63 – 7.

58. Zhang B, Gojo I, Fenton RG. Myeloid cell factor-1 is a critical survival factor for multiple myeloma. Blood. 2002;99:1885 – 93.

59. Pagnano KB, Silva MD, Vassallo J, Aranha FJ, Saad ST. Apoptosis-regulating proteins and prognosis in diffuse large B cell non-Hodgkin's lymphomas. Acta Haematol. 2002;107:29 – 34.

60. Rassidakis GZ, Lai R, McDonnell TJ, Cabanilla F, Sarris AH, Mederios LJ. Overexpression of Mcl-1 in anaplastic large cell lymphoma cell lines and tumors. Am J Pathol. 2002;160:2309 – 10.

61. Derenne S, Monia B, Dean NM, Taylor JK, Rapp MJ, Harousseau JL, et al. Antisense strategy shows that Mcl-1 rather than Bcl-2 or Bcl-x(L) is an essential protein of human myeloma cells. Blood. 2002;100:194 – 9.

62. Byrd JC, Kitada S, Flinn IW, Aron JL, Pearson M, Lucas D, et al. The mechanism of tumor cell clearance by rituximab *in vivo* in patients with B-cell chronic lymphocytic leukemia: evidence of caspase activation and apoptosis induction. Blood. 2002;99:1038 – 43.

63. Poruchynsky MS, Giannakakou P, Ward Y, Bulinski JC, Telford WG, Robey RW, et al. Accompanying protein alterations in malignant cells with a microtubule-polymerizing drug-resistance phenotype and a primary resistance mechanism. Biochem Pharmacol. 2001;62: 1469 – 80.

64. Huang HM, Huang CJ, Yen JJ. Mcl-1 is a common target of stem cell factor and IL-5 for apoptosis prevention activity via MEK/MAPK and PI-3K/AKT pathways. Blood. 2000;96:1764 – 71.

65. Schubert KM, Duroino V. Distinct roles of extracellular-signal-regulated protein kinase (ERK) mitogen activated protein kinases and phosphatidylinositol 3-kinase in the regulation of Mcl-1 synthesis. Biochem J. 2001;356:473 – 80.

66. MacKeigan JP, Collins TS, Ting JP-Y. MEK inhibition enhances paclitaxel-induced tumor apoptosis. J Biol Chem. 2000;275:38953 – 6.

67. Dai Y, Yu C, Sing V, Tang L, Wang Z, Molinstry R, et al. Pharmacological inhibitors of the mitogen activated protein kinase (MAPK) kinase/MAPK cascade interact synergistically with UCN-01 to induce mitochondrial dysfunction and apoptosis in human leukemia cells. Cancer Res. 2001;61:5106 – 15.

68. Roy N, Deveraux QL, Takahashi R, Salvesen GS, Reed JC. The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases. EMBO J. 1997;16:6914 – 25.

69. Deveraux QL, Roy N, Stennicke HR, Arsdale TV, Zhou Q, Srinivasula SM, et al. IAPs block apoptotic events induced by caspase-8 and cytochrome *c* by direct inhibition of distinct caspases. EMBO J. 1998;17:2215 – 23.

70. Bernardi P, Scorrano L, Colonna R, Petronilli V, Di Lisa F. Mitochondria and cell death. Mechanistic aspects and methodological issues. Eur J Biochem. 1999;264:687 – 701.

71. Scorrano L, Petronilli V, Colonna R, Di Lisa F, Bernardi P. Chloromethyl-tetramethylrosamine (Mitotracker Orange) induces the mitochondrial permeability transition and inhibits respiratory complex I. Implications for the mechanism of cytochrome *c* release. J Biol Chem. 1999;274:24657 – 63.

72. Scorrano L, Ashiya M, Buttle K, Weiler S, Oakes SA, Mannella CA, et al. A distinct pathway remodels mitochondrial cristae and mobilizes cytochrome *c* during apoptosis. Dev Cell. 2002;2:55 – 67.

73. Waterhouse NJ, Ricci JE, Green DR. And all of a sudden it's over: mitochondrial outer-membrane permeabilization in apoptosis. Biochemie. 2002;84:113 – 21.

Molecular Cancer Therapeutics

Resveratrol modifies the expression of apoptotic regulatory proteins and sensitizes non-Hodgkin's lymphoma and multiple myeloma cell lines to paclitaxel-induced apoptosis

Ali R. Jazirehi and Benjamin Bonavida

Mol Cancer Ther 2004;3:71-84.