

Resveratrol Exerts Antiproliferative Activity and Induces Apoptosis in Waldenström's Macroglobulinemia

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Abstract Purpose: Resveratrol (3,4',5-tri-hydroxy-*trans*-stilbene) is an antioxidant constituent of a wide variety of plant species including grapes. It has gained considerable attention because of its anticancer properties, as shown in solid and hematologic malignancies. Whether resveratrol could inhibit proliferation or induce cytotoxicity in Waldenström's macroglobulinemia (WM) was investigated.

Experimental Design: We studied resveratrol-induced inhibition of proliferation and induction of cytotoxicity in WM cell lines, WM primary tumor cells, IgM-secreting cells, and peripheral blood mononuclear cells. The mechanisms of action and different signaling pathways involved were studied using Western blot and gene expression profile analysis. Resveratrol activity was also evaluated in the bone marrow microenvironment. We finally investigated whether or not resveratrol could have any synergistic effect if used in combination with other drugs widely used in the treatment of WM.

Results: Resveratrol inhibited proliferation and induced cytotoxicity against WM cells, IgM-secreting cells, as well as primary WM cells, without affecting peripheral blood mononuclear cells; down-regulated Akt, extracellular signal-regulated kinase mitogen-activated protein kinases, and Wnt signaling pathways, as well as Akt activity; induced cell cycle arrest and apoptosis; and triggered *c-Jun*-NH₂-terminal-kinase activation, followed by the activation of intrinsic and extrinsic caspase pathways. Lastly, adherence to bone marrow stromal cells did not confer protection to WM cells against resveratrol-induced cytotoxicity. Furthermore, resveratrol showed synergistic cytotoxicity when combined with dexamethasone, fludarabine, and bortezomib.

Conclusion: Our data show that resveratrol has significant antitumor activity in WM, providing the framework for clinical trials in this disease.

Waldenström's macroglobulinemia (WM) is a low-grade lymphoproliferative disorder characterized by the presence of a lymphoplasmacytic infiltrate in the bone marrow and the presence of a serum monoclonal protein IgM (1, 2).

Despite the clinical efficacy of conventional therapies including alkylating agents, nucleoside analogues, steroids, rituximab, as well as novel agents including alemtuzumab and

bortezomib (3, 4), most patients eventually relapse and the disease remains incurable. Therefore, new therapeutic strategies are needed in order to improve patient outcome.

The polyphenolic phytoalexin *trans*-resveratrol (3,4',5-tri-hydroxy-*trans*-stilbene) is an antioxidant constituent of a wide variety of plant species including grapes, and it is present in red wine (5). It has recently gained considerable attention because of its potential cancer chemopreventive or anticancer properties (6). Moreover, it may be beneficial in the prevention of cardiovascular diseases, arthritis or autoimmune disorders, particularly when used at low concentrations (7, 8). However, when administered at higher doses, resveratrol possesses anticancer activity by interfering with different cellular events related to the initiation, promotion, and progression of multistage carcinogenesis (9). Previous reports have shown that resveratrol induces apoptosis and inhibits the proliferation of several solid tumor cell lines including osteosarcoma (SJS), uterine (HeLa, HEC-1A, RL95-2, KLE, EN-1078D), breast (MCF-7, MDA-MB-231), and colon cancer (HT29), as well as hematologic malignancies including Burkitt's lymphoma (HS-Sultan), promyelocytic leukemia (HL-60), and multiple myeloma (IM9, U266, RPMI 8226; refs. 10–17). It has also been shown that resveratrol could interfere with apoptotic pathways

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both by directly triggering apoptosis-promoting signaling cascades and by blocking antiapoptotic mechanisms. By blocking survival and apoptotic pathways, resveratrol could sensitize cancer cells, which may result in synergistic antitumor activities when resveratrol is combined with conventional chemotherapeutic agents or cytotoxic compounds.

The possible anticancer potential of resveratrol in WM, however, remains unknown. In this study, we evaluated the therapeutic potential of resveratrol against WM. We showed that resveratrol inhibited growth and induced apoptosis in WM cells via both caspase-dependent and -independent pathways. Moreover, resveratrol down-regulated the Wnt signaling pathway with a reduction of nuclear β -catenin levels and decrease of the protein expression of myc and survivin, both downstream target proteins of β -catenin. Resveratrol also overcame bone marrow-induced resistance and enhanced WM cell cytotoxicity by bortezomib, dexamethasone, and fludarabine.

Materials and Methods

Cells. The WM cell lines (BCWM.1; WM-WSU) and IgM-secreting low-grade lymphoma cell lines (MEC-1; RL) were used in this study. BCWM.1 is a recently described WM cell line that was developed from a patient with untreated WM (18). WSU-WM was kindly provided by Dr. Al Katib (Wayne State University, Detroit, MI). MEC-1 was a gift from Dr. Kay (Mayo Clinic, Rochester, MN). RL was purchased from the American Tissue Culture Collection. All cell lines were cultured at 37°C in RPMI 1640 containing 10% fetal bovine serum (Sigma Chemical), 2 mmol/L of L-glutamine, 100 units/mL of penicillin, and 100 μ g/mL of streptomycin (Life Technologies).

Primary WM cells were obtained from bone marrow (BM) samples from previously treated WM patients, using CD19⁺ microbead selection (Miltenyi Biotec) with >90% purity as confirmed by flow cytometric analysis with monoclonal antibody reactive to human CD20-PE (BD-Bioscience). Peripheral blood mononuclear cells (PBMC) were obtained from healthy subjects by Ficoll-Hypaque density sedimentation. Cells were cultured at 37°C in RPMI 1640 containing 10% fetal bovine serum (Sigma Chemical), 2 mmol/L of L-glutamine, 100 units/mL of penicillin, and 100 μ g/mL of streptomycin (Life Technologies). Approval for these studies was obtained from the Dana-Farber Cancer Institute Institutional Review Board. Informed consent was obtained from all patients and healthy volunteers in accordance with the Declaration of Helsinki protocol.

Reagents. Resveratrol, with a purity of >99%, was purchased from Sigma. A 20 mmol/L stock solution of resveratrol was dissolved in DMSO and stored at -20°C until use; it was diluted in culture medium (5-80 μ mol/L) immediately before use. The maximum final concentration of DMSO (<0.1%) did not affect cell proliferation and did not induce cytotoxicity on all the cell lines and primary cells tested (data not shown). Bortezomib was obtained from Millennium Pharmaceuticals, Inc. Fludarabine and dexamethasone were purchased from Sigma. The *c-Jun*-NH₂-kinase (JNK) inhibitor SP600215 was purchased from Calbiochem.

Growth inhibition assay. The inhibitory effect of resveratrol, alone or combined with other agents on WM cell growth, was assessed by CalceinAM assay. CalceinAM is a nonfluorescent, hydrophilic compound that permeates intact, live cells. The hydrolysis of CalceinAM by intracellular esterases produces calcein, a hydrophilic, strongly fluorescent compound that is well-retained in the cell cytoplasm. Briefly, WM cells and PBMCs (3×10^4 cells/well) were incubated in 96-well culture plates (Costar) in the presence of RPMI (10% fetal bovine serum) with resveratrol (0-80 μ mol/L), alone or in combination with other agents for 24, 48, and 72 h at 37°C, and then

washed twice using $1 \times$ CalceinAM DW Buffer. They were subsequently incubated with CalceinAM (2 μ mol/L) for 30 min at 37°C under CO₂. The fluorescence intensity, proportional to the number of viable cells, was recorded using a multilabel reader (490 nm excitation filter, 520 nm emission filter), Mithras LB940 (Berthold Technologies).

DNA synthesis. WM cell lines and CD19⁺ primary WM cells were incubated in the presence of RPMI (10% fetal bovine serum) with resveratrol (0-80 μ mol/L), alone or in combination with other agents for 24, 48, and 72 h at 37°C. DNA synthesis was measured by [³H]thymidine ([³H]TdR; Perkin-Elmer) uptake, as previously described (19). Cells were pulsed with [³H]TdR, 0.0185 MBq/well (0.5 μ Ci/well) during the last 8 h (cell lines) or 24 h (CD19⁺ primary WM cells) of 24-, 48-, or 72-h cultures. All experiments were done in triplicate.

Immunoblotting. BCWM.1 cells were harvested and lysed using lysis buffer (Cell Signaling Technology) reconstituted with 5 mmol/L of NaF, 2 mmol/L of Na₃VO₄, 1 mmol/L of polymethylsulfonyle fluoride, 5 μ g/mL of leupeptine, and 5 μ g/mL of aprotinin. Whole cell lysates (50 μ g/lane) were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membrane (Bio-Rad Laboratories). The antibodies used for immunoblotting included anti-phosphorylated Akt (Ser⁴⁷³), anti-Akt, anti-phosphorylated GSK3 α - β (Ser^{21/9}), anti-phosphorylated ERK1/2 (Thr²⁰²/Tyr²⁰⁴), anti-ERK1/2, anti-caspase-3, anti-caspase-8, anti-caspase-9, anti-PARP, anti-phosphorylated JNK (Thr¹⁸³/Tyr¹⁸⁵), anti-JNK, anti-cdk2, anti-cdk4, anti-cdk6, anti-phosphorylated Rb (Ser^{807/811}), anti-Rb, anti-p63, anti-p73, anti-p21, anti-p27, anti-cyclin D1, anti-cyclin D2, anti-mcl1, anti-survivin, and anti-phosphorylated S6 ribosomal (Cell Signaling Technology); anti-myc (BD Biosciences); anti-cyclin E1 (AbCam); and anti-p53 and anti- α -tubulin antibodies (Santa Cruz Biotechnology). Nuclear extracts of the cells were prepared using the Nuclear Extraction Kit (Panomics, Inc.) and subjected to immunoblotting with anti- β -catenin and anti-nucleolin antibodies (Santa Cruz Biotechnology).

In vitro Akt kinase assay. *In vitro* Akt kinase assay (Cell Signaling Technology) was done as previously described (19). Briefly, BCWM.1 cells were cultured in the presence or absence of resveratrol (2.5-60 μ mol/L, for 6 h) and subsequently lysed in $1 \times$ lysis buffer. Lysates were then immunoprecipitated with immobilized Akt primary antibody and incubated with gentle rocking overnight at 4°C. Cell lysate/immobilized antibody were microcentrifuged and pellets were washed twice with $1 \times$ cell lysis buffer, and twice with $1 \times$ kinase buffer. Pellets were resuspended in $1 \times$ kinase buffer supplemented with ATP and GSK-3 fusion protein, and then incubated for 30 min at 30°C. Samples were run on SDS-PAGE and transferred to polyvinylidene difluoride membrane. Kinase activity was detected by immunoblotting with phosphorylated GSK-3 α / β (Ser^{21/9}) antibody (Cell Signaling).

Effect of resveratrol on paracrine WM cell growth in the BM. To evaluate growth stimulation and signaling in WM cells adherent to bone marrow stromal cells (BMSC), 3×10^4 BCWM.1 cells were cultured in BMSC-coated 96-well plates for 48 h in the presence or absence of resveratrol. DNA synthesis was measured as described (19).

Cell cycle analysis. WM cells cultured for 24 h with resveratrol or control medium were harvested, washed with PBS, fixed with 70% ethanol, and treated with 10 μ g/mL of RNase (Roche Diagnostic). Cells were then stained with propidium iodide (PI; Sigma Chemical; 5 μ g/mL) and the cell cycle profile was determined using an Epics Altra flow cytometer (Coulter Immunology) as described (19).

Detection of apoptosis. Annexin V-FITC and PI staining were used to detect and quantify apoptosis by flow cytometry. BCWM.1 cells (1×10^6 cells/well) were cultured in 24-well plates (Costar) for 24 h with resveratrol (5-20 μ mol/L) or control medium. Cells were then harvested in cold PBS and pelleted by centrifugation for 5 min at 1,500 rpm. They were subsequently resuspended at 1×10^6 cells/mL in binding buffer [Hepes buffer, 10 mmol/L (pH 7.4), 150 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgCl₂, 1.8 mmol/L CaCl₂], stained with Annexin V-FITC and PI, and incubated in the dark for 15 min. Cells were processed with an Epics Altra flow cytometer (Coulter Immunology).

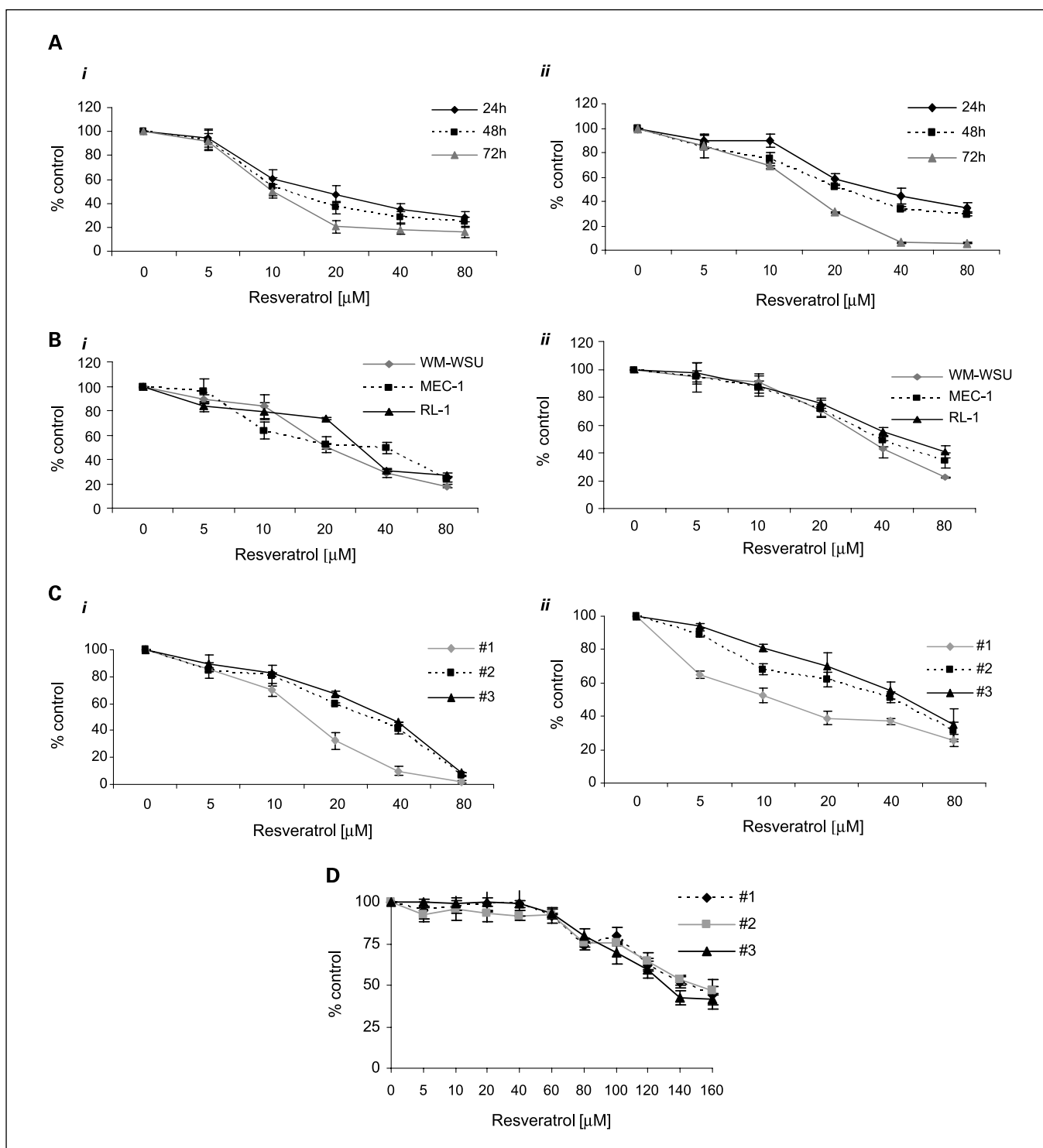


Fig. 1. Resveratrol induces a decrease in DNA synthesis and triggers cytotoxicity in WM cells. BCWM.1 cells were cultured with resveratrol (5–80 μ mol/L) for 24 h (\blacklozenge), 48 h (\blacksquare), and 72 h (\blacktriangle). DNA synthesis was assessed by thymidine uptake (A) and cytotoxicity was assessed by CalceinAM uptake (D). Several IgM-secreting cell lines, WM-WSU (\blacklozenge), MEC-1 (\blacksquare), and RL-1 (\blacktriangle) were cultured for 48 h with resveratrol (5–80 μ mol/L). DNA synthesis was assessed by thymidine uptake (B) and cytotoxicity was assessed by CalceinAM uptake (E). Freshly isolated bone marrow CD19⁺ tumor cells from three patients with WM. DNA synthesis was assessed by thymidine uptake (C) and cytotoxicity was assessed by CalceinAM uptake (F) after 48 h of exposure to resveratrol. PBMCs from three healthy donors were cultured with resveratrol (5–160 μ mol/L) for 48 h. Cytotoxicity was assessed by CalceinAM uptake (G). Control represents untreated cells. Points, mean; bars, SD. All experiments were done in triplicate.

Gene expression profile. BCWM.1 cells, untreated or treated with 20 μ mol/L of resveratrol for 6 and 24 h, were harvested and total RNA was isolated using an RNeasy kit (Qiagen) as described by the manufacturer. Total RNA (15 μ g) was reverse-transcribed to get cDNA

using the Superscript II reverse transcription kit (Invitrogen Life Technologies). cDNA was used in an *in vitro* transcription reaction to synthesize biotin-labeled cRNA using the Enzo biotinylated nucleotides bio-UTP and bio-CTP along with the Ambion T7 Megascript Kit (Enzo

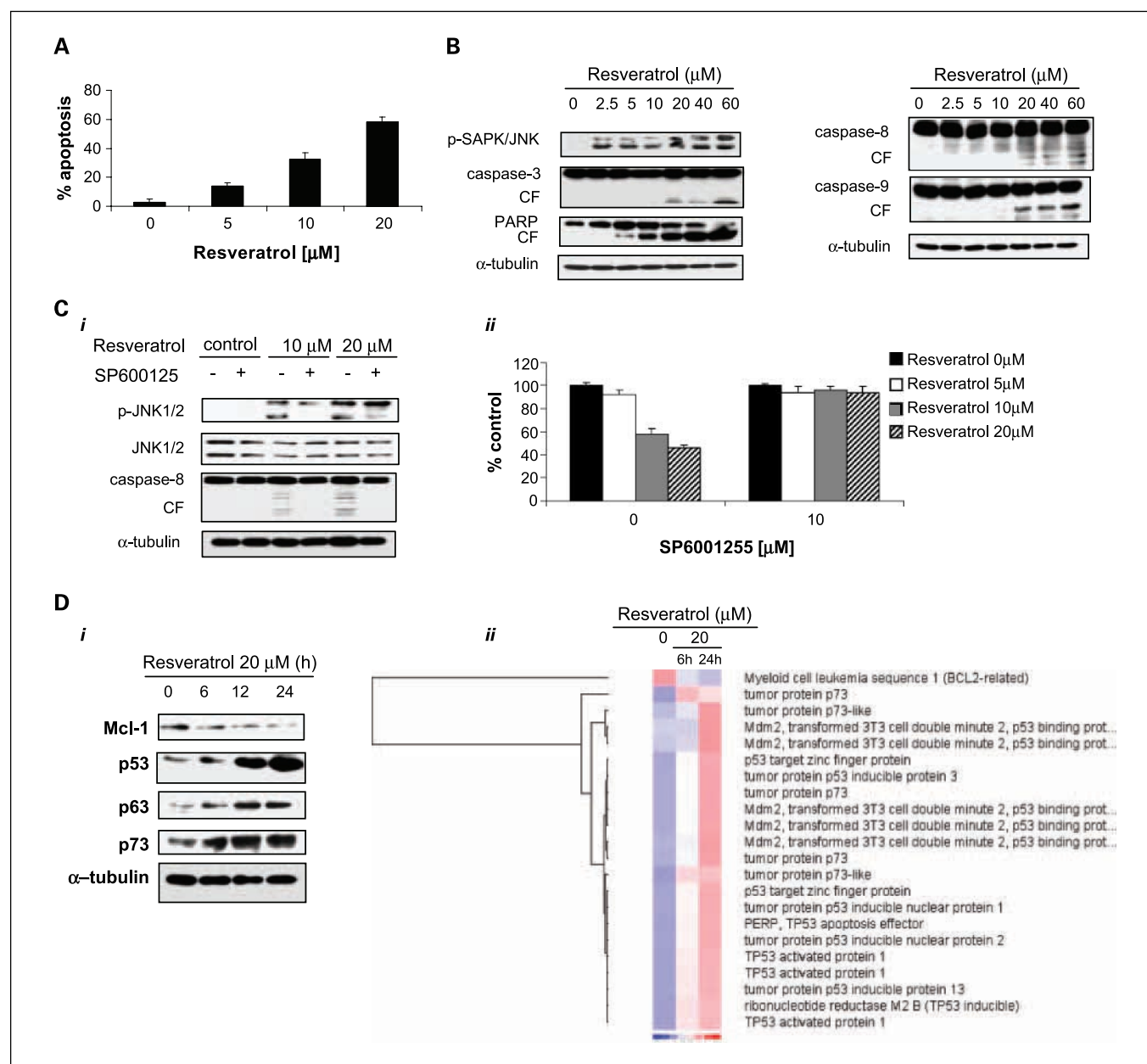


Fig. 2. Resveratrol induces apoptosis in the WM cell line BCWM.1 and modulates the expression of Mcl-1 and p53 family members. **A**, BCWM.1 were cultured with resveratrol for 24 h at doses ranging from 5 to 20 μmol/L, and the percentage of cells undergoing apoptosis was studied by Annexin V and PI staining at 24 h. **B**, BCWM.1 cells were cultured with resveratrol (2.5-60 μmol/L) for 24 h. Whole cell lysates were subjected to Western blotting using anti-caspase-8, anti-caspase-9, anti-caspase-3, anti-PARP, anti-phosphorylated JNK1/2, and anti-α-tubulin antibodies. **C**, BCWM.1 were cultured with resveratrol (10-20 μmol/L) for 24 h, in the presence or absence of SP600125 (10 μmol/L). Cells were then lysed and subjected to Western blotting using anti-phosphorylated JNK1/2, anti-JNK1/2, anti-caspase 8, and anti-α-tubulin antibodies. **D**, BCWM.1 cells were cultured for 24 h with control medium, or with resveratrol (5, 10, and 20 μmol/L), in presence or absence of SP600125 (10 μmol/L). **E**, BCWM.1 were cultured with resveratrol (20 μmol/L) for 6, 12, and 24 h. Whole cell lysates were subjected to Western blotting using anti-Mcl-1, anti-p53, anti-p63, anti-p73, and anti-α-tubulin antibodies. **F**, BCWM.1 cells were cultured in the absence or presence of resveratrol (20 μmol/L) for 6 and 24 h. Purified cRNA (15 μg) was hybridized to HG-U133Plus2.0 GeneChip (Affymetrix). Fold change in the expression of resveratrol-treated cells relative to expression in untreated BCWM.1 cells is shown by the intensity of induction (red) or suppression (blue).

Diagnostic and Applied Biosystems). Labeled cRNA was purified with the RNeasy mini-kit (Qiagen) and quantitated. Purified cRNA (15 μg) was hybridized to Human Genome U133Plus2.0 (HG-U133Plus2.0) GeneChip arrays (Affymetrix) according to the manufacturer's protocol. The HG-U133Plus2.0 array analyzes the expression level of >47,000 transcripts and variants, including 38,500 well-characterized human genes. The array is comprised of >54,000 probe sets and 1,300,000 distinct oligonucleotide features. GeneChip arrays were scanned on a GeneChip Scanner 3000 (Affymetrix).

The normalization of arrays and calculation of expression values was done using the DNA-chip analyzer (dChip) program (20, 21). Arrays were normalized based on relative signal produced for an invariant subset of genes. This model-based method was used for probe selection and computing expression values (20, 21). By pooling hybridization information across multiple arrays, it is possible to assess standard errors for the expression level indexes. We also used several high-level analysis functions in dChip for comparative analysis and hierarchical clustering.

Isobologram analysis. The interaction between resveratrol and other agents was analyzed by isobologram analysis using the CalcuSyn software program (Biosoft) to determine if the combinations were additive or synergistic. This program is based on the Chou-Talalay method, which calculates a combination index (CI) to indicate additive or synergistic effects. When CI = 1, the effects were additive, when CI < 1.0, the effects were synergistic. Results from viability assay (CalceinAM uptake) were expressed as the fraction of cells killed by the single drug or the combination in drug-treated versus untreated cells (19).

Statistical analysis. The statistical significance of differences in drug-treated versus control cultures was determined using Student's *t* test. The minimal level of significance was $P < 0.05$.

Results

Resveratrol inhibits DNA synthesis and induces cytotoxicity of WM cells. WM and IgM-secreting cell lines were cultured for 24, 48, and 72 h in the presence or absence of resveratrol (5-80 $\mu\text{mol/L}$). Resveratrol inhibited BCWM.1 proliferation as measured by [^3H]TdR uptake assay, with an IC_{50} between

10 and 15 $\mu\text{mol/L}$ (Fig. 1A). Resveratrol showed similar antiproliferative activity on all IgM-secreting cell lines tested with an IC_{50} between 20 and 30 $\mu\text{mol/L}$ (Fig. 1B). The antiproliferative activity of resveratrol against primary lymphoplasmacytic cells from three patients with WM was also evaluated, wherein resveratrol decreased DNA synthesis, as measured by [^3H]TdR at 48 h, in a dose-dependent fashion, with an IC_{50} between 15 and 32 $\mu\text{mol/L}$ (Fig. 1C).

We next evaluated the potential cytotoxic effect of resveratrol (5-80 $\mu\text{mol/L}$) on WM cell lines and primary WM cells. Because it has been previously reported that resveratrol could modulate 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrasodium bromide—reducing activity independently of cell killing (22), we assessed the cytotoxic effect of resveratrol by CalceinAM uptake. Resveratrol triggered cytotoxicity in BCWM.1 WM cells at 24, 48, and 72 h with an IC_{50} between 15 and 32 $\mu\text{mol/L}$ (Fig. 1D), in IgM-secreting cells at 48 h with an IC_{50} between 20 and 30 $\mu\text{mol/L}$ (Fig. 1E), and in primary lymphoplasmacytic cells from three patients

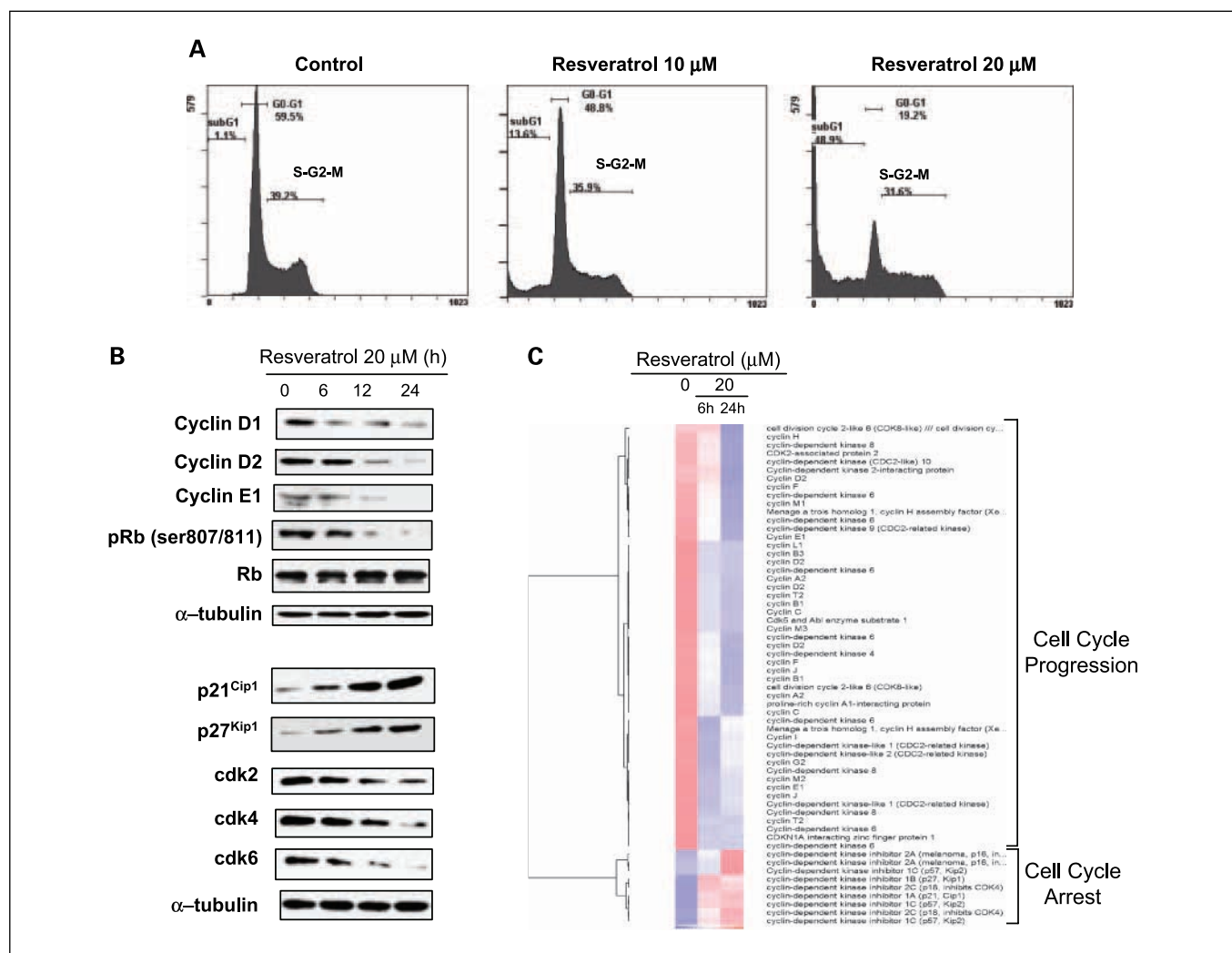


Fig. 3. Resveratrol induces cell cycle arrest on WM cell line BCWM.1. **A**, BCWM.1 were cultured with resveratrol (10-20 $\mu\text{mol/L}$) for 24 h. Cells were then subjected to cell cycle profiling by PI staining and flow cytometry. **B**, BCWM.1 cells were cultured in the absence or presence of resveratrol (10 $\mu\text{mol/L}$) for the times indicated. Cells were then lysed and subjected to Western blotting using anti-p21^{Cip1}, anti-p27^{Kip1}, anti-cyclin D1, anti-cyclin D2, anti-cyclin E1, anti-cdk2, anti-cdk4, anti-cdk6, anti-phosphorylated Rb, anti-Rb, and anti- α -tubulin antibodies. **C**, BCWM.1 cells were cultured in the absence or presence of resveratrol (20 $\mu\text{mol/L}$) for 6 and 24 h. Purified cRNA (15 μg) was hybridized to HG-U133Plus2.0 GeneChip (Affymetrix). Fold change in the expression of resveratrol-treated cells relative to expression in untreated BCWM.1 cells is shown by the intensity of induction (red) or suppression (blue).

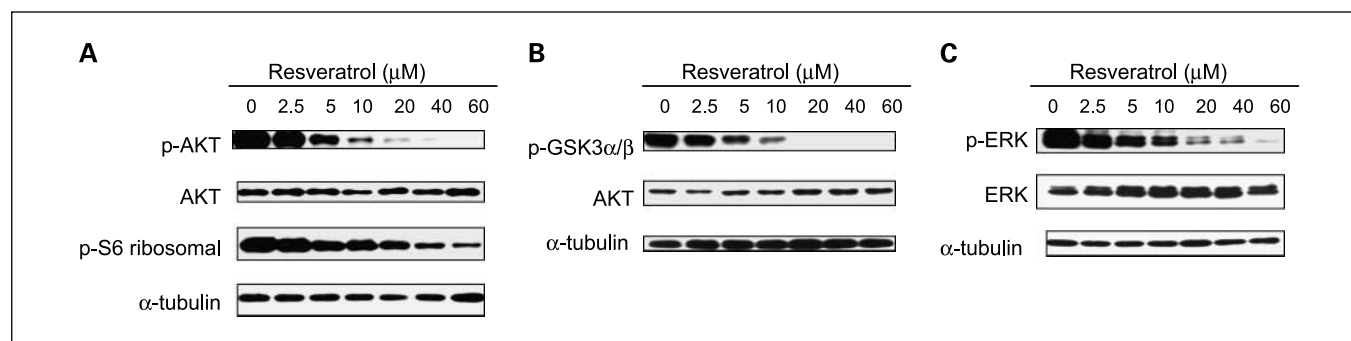


Fig. 4. Resveratrol modulates growth signaling pathways in BCWM.1. *A*, BCWM.1 were cultured with resveratrol (2.5–60 $\mu\text{mol/L}$) for 8 h. Whole cell lysates were subjected to Western blotting using anti-phosphorylated AKT, anti-AKT, anti-phosphorylated S6 ribosomal and anti- α -tubulin antibodies. *B*, Akt kinase assay. BCWM.1 cells were cultured with control medium or resveratrol (2.5–60 $\mu\text{mol/L}$) for 6 h. Whole cell lysates were immunoprecipitated with anti-Akt antibody. Then the immunoprecipitate was washed and subjected to *in vitro* kinase assay according to the manufacturer's protocol. Western blotting used anti-phosphorylated GSK3 α/β and anti-Akt antibodies. *C*, BCWM.1 were cultured with resveratrol (2.5–60 $\mu\text{mol/L}$) for 8 h. Whole cell lysates were subjected to Western blotting using anti-phosphorylated ERK, anti-ERK1/2, and anti- α -tubulin antibodies.

with WM between 10 and 40 $\mu\text{mol/L}$ (Fig. 1F). In contrast, resveratrol did not induce cytotoxicity in PBMCs isolated from three healthy volunteers at the examined dose ranges (Fig. 1G). These results indicate that resveratrol triggers significant cytotoxicity in WM cells at concentrations that are not appreciably cytotoxic to normal PBMCs.

Resveratrol induces apoptosis in WM cells. We examined the molecular mechanisms whereby resveratrol induces cytotoxicity in WM cells. We showed that resveratrol induced dose-dependent apoptosis, as evidenced by Annexin V and PI staining and flow cytometry analysis. The percentage of apoptotic BCWM.1 cells increased from 2.23% (untreated) to 13.48%, 30.89%, and 57.64% after 24 h of treatment with 5, 10, and 20 $\mu\text{mol/L}$ of resveratrol, respectively (Fig. 2A). Similar data were obtained on other IgM-secreting cell lines (data not shown). To determine the mechanism of resveratrol-induced apoptosis, we investigated the effect of resveratrol on BCWM.1 cells using immunoblotting. BCWM.1 cells were treated with increasing doses of resveratrol (2.5–60 $\mu\text{mol/L}$) for 24 h. Resveratrol triggered JNK activation, followed by caspase-3, caspase-8, caspase-9, and PARP cleavage (Fig. 2B). Because it has been shown that JNK plays a pivotal role in apoptosis triggered by several novel therapeutic agents including bortezomib (23), lysophosphatidic acyltransferase inhibitor, and perifosine (19), we sought to clarify if resveratrol induced the phosphorylation of JNK during WM cell apoptosis. We found that resveratrol induced the phosphorylation of JNK1/2 in BCWM.1 cells in a dose-dependent manner (Fig. 2C). To determine the role of JNK activity in mediating resveratrol-induced cytotoxicity, BCWM.1 were treated with resveratrol in the presence or absence of the JNK inhibitor SP600125 (24). SP600125 markedly inhibited resveratrol-induced phosphorylation of JNK and caspase-8 cleavage (Fig. 2C). Moreover, we showed that cytotoxicity triggered by resveratrol (5, 10, and 20 $\mu\text{mol/L}$) was blocked by SP600125 (Fig. 2D), confirming that SP600125 inhibits resveratrol-induced JNK activation and caspase-8 cleavage.

Moreover, we observed that resveratrol induced the up-regulation of the proapoptotic transcription factor p53 and down-regulated the antiapoptotic protein Mcl-1 in a time-dependent manner (Fig. 2E). In addition, we found that resveratrol induced the up-regulation of p63 and p73,

members of the p53 family with proapoptotic ability (Fig. 2E; ref. 25). Importantly, resveratrol-induced protein modulation for p53, p63, p73, and Mcl-1 was also confirmed by gene expression analyses (Fig. 2F), providing further validation of resveratrol's effect on these crucial apoptotic regulatory molecules.

We next examined the cell cycle profiling using PI staining in BCWM.1 cells cultured for 24 h with control medium or resveratrol (10 and 20 $\mu\text{mol/L}$). Resveratrol induced cell cycle arrest, as shown by a decrease of G₀ to G₁ phase cells, from 48.8% to 19.2%; indeed, cell cycle arrest induced cell death as shown by an increase of sub-G₀ to sub-G₁ phase cells, from 13.6% to 48.9%, using 10 and 20 $\mu\text{mol/L}$ of resveratrol, respectively. To determine the mechanism of resveratrol-induced cell cycle arrest, we investigated the effect of resveratrol on BCWM.1 cells using immunoblotting. BCWM.1 cells were treated with resveratrol (20 $\mu\text{mol/L}$) for 6, 12, and 24 h. Resveratrol induced the up-regulation of cyclin kinase inhibitor proteins p21^{Cip1} and p27^{Kip1}, and the down-regulation of cyclin D1, D2, E1, as well as cyclin-dependent kinase (cdk2, cdk4, and cdk6) protein levels (Fig. 3B). In addition, resveratrol reduced the phosphorylation of Rb which prevented G₁-S cell cycle progression resulting in cell cycle arrest (Fig. 3B; ref. 26). Importantly, the results of resveratrol-induced protein modulation were also confirmed by gene expression analysis (Fig. 3C), providing further validation of resveratrol's effect on these important regulators of the cell cycle.

Signaling pathways regulated by resveratrol. It has been shown that phosphatidylinositol-3-kinase (PI3K)/Akt pathway plays a pivotal role in promoting growth and survival of tumor B cells (27). We therefore investigated whether or not resveratrol could affect PI3K/Akt signaling pathway in WM cells. BCWM.1 cells were treated with increasing doses of resveratrol (2.5–60 $\mu\text{mol/L}$) for 8 h. We found that resveratrol inhibited the phosphorylation of Akt (Ser⁴⁷³) and downstream ribosomal protein S6 in a dose-dependent manner at 8 h (Fig. 4A). Moreover, Akt kinase activity assay decreased the phosphorylation of GSK3 fusion protein after treatment with resveratrol in a dose-dependent fashion at 6 h (Fig. 4B). We next investigated the effect of resveratrol on the MEK/ERK pathway, which is known to increase growth and to enhance survival of tumor B cells (28, 29). By Western blot analysis, we observed that

resveratrol decreased phosphorylation of ERK1/2 (Thr²⁰²/Tyr²⁰⁴) at 8 hours in a dose-dependent manner (Fig. 4C).

Resveratrol affects the β -catenin/TCF pathway and inhibits the expression of β -catenin/TCF pathway downstream target genes. Previous studies have shown that Wnt signaling genes are overexpressed in B-cell malignancies, including B-cell chronic lymphocytic leukemia and multiple myeloma (30, 31). The classic Wnt signaling pathway increases the β -catenin translocation to the nucleus, and the transcription of target genes, including *myc* and *survivin*, responsible for cell cycle progression, cell survival, cell proliferation, and disease progression (30, 31). Therefore, we investigated whether or not resveratrol could modulate the transcript level of genes related to the Wnt signaling cascade in the WM cell line. Gene expression analysis on BCWM.1 cultured in the presence or absence of resveratrol (20 μ M/L; 6 and 24 h) was done. We found that resveratrol down-regulated the transcript levels of Wnt signaling genes in BCWM.1 as well as the transcript levels of β -catenin, *myc* and *survivin*, both downstream target genes in the β -catenin/TCF pathway (Fig. 5A; refs. 32, 33). Moreover, we confirmed the observed changes in gene expression profiling at the protein level using immunoblotting: resveratrol (10 and 20 μ M/L)

down-regulated nuclear β -catenin levels in BCWM.1 cells, whereas total β -catenin levels remained unaffected (Fig. 5B); resveratrol also down-regulated the protein expression of *myc* and *survivin* (Fig. 5C).

Adherence to BMSCs do not protect against resveratrol-induced WM cytotoxicity. Because the BM microenvironment confers growth and induces drug resistance in malignant cells (34), we next investigated whether resveratrol inhibits WM cell growth in the context of the BM milieu. BCWM.1 cells were cultured with resveratrol (5-20 μ M/L) in the presence or absence of BMSCs. Using [³H]TdR uptake assay, adherence of BCWM.1 cells to BMSCs triggered an increase of 30% in proliferation, which was inhibited by resveratrol in a dose-dependent manner (Fig. 6A). The viability of BMSCs, assessed by CalceinAM uptake and trypan blue exclusion, was not affected by resveratrol treatment (data not shown). The adherence of BCWM.1 to BMSCs, however, induced Akt and ERK phosphorylation in BCWM.1 cells, which was inhibited by resveratrol (Fig. 6B). These data indicate that resveratrol may trigger significant antitumor activity against WM cells even in the presence of the BM milieu.

Resveratrol enhances cytotoxicity exerted by other agents active in WM. We also sought to investigate the effects of resveratrol

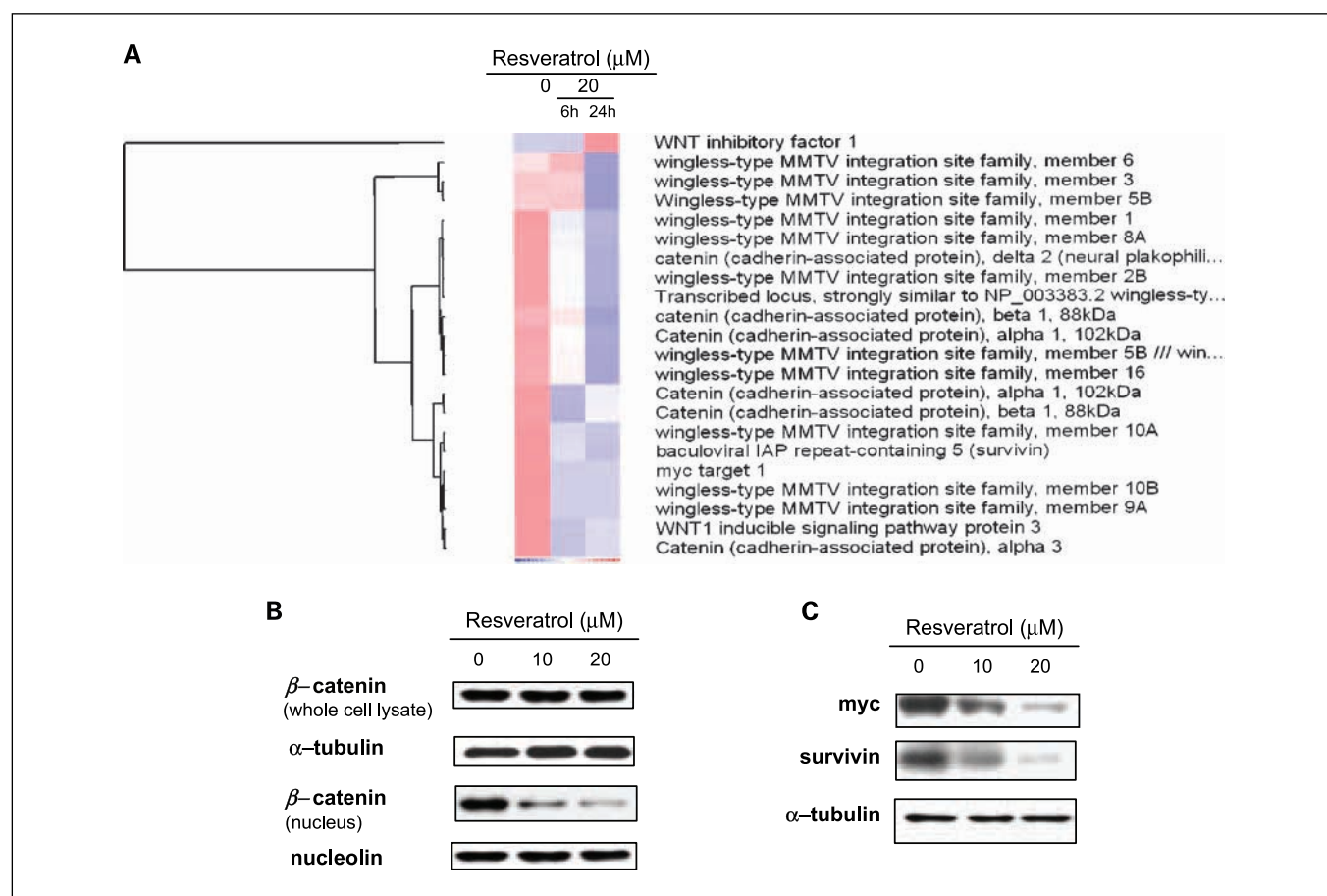


Fig. 5. Resveratrol down-regulates Wnt signaling pathway. **A**, BCWM.1 cells were cultured in the absence or presence of resveratrol (20 μ M/L) for 6 and 24 h. Purified cRNA (15 μ g) was hybridized to HG-U133Plus2.0 GeneChip (Affymetrix). Fold change in the expression in resveratrol-treated cells relative to expression in untreated BCWM.1 cells was shown by the intensity of induction (red) or suppression (blue). **B**, BCWM.1 were cultured with resveratrol (10-20 μ M/L) for 6 h. Total cell lysates and nuclear extracts were subject to immunoblotting using anti- β -catenin and anti- α -tubulin antibodies. Nuclear extracts were subject to immunoblotting using anti- β -catenin and anti-nucleolin antibodies. **C**, BCWM.1 were cultured with resveratrol (10-20 μ M/L) for 6 h. Total cell lysates were subject to immunoblotting using anti-*myc*, anti-*survivin*, and anti- α -tubulin antibodies.

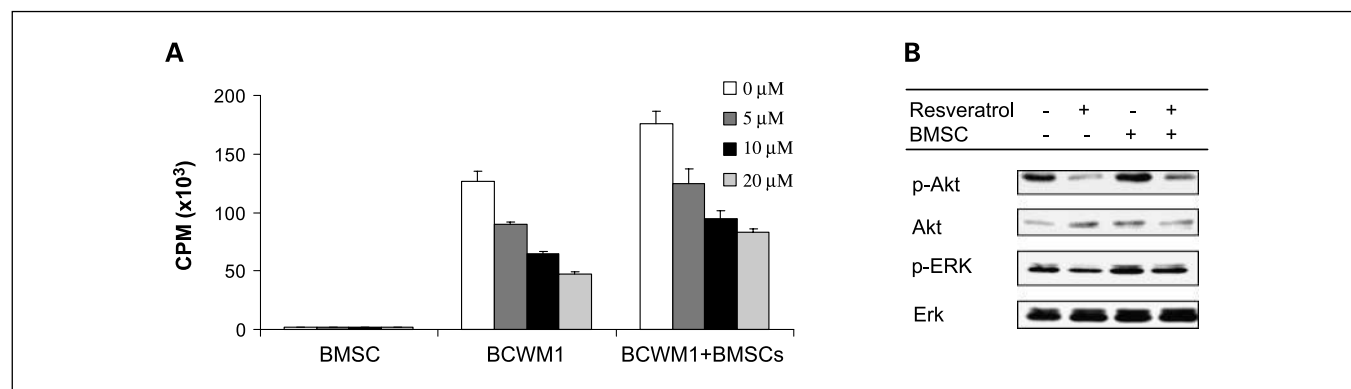


Fig. 6. Adherence to BMSCs does not protect against resveratrol-induced WM cell cytotoxicity. **A**, BCWM.1 cells were cultured with control medium, and with resveratrol (5, 10, and 20 μmol/L) for 48 h, in the presence or absence of BMSCs. Cell proliferation was assessed using [³H]TdR uptake assay. Columns, mean of triplicate experiments; bars, SD. **B**, BCWM.1 were cultured with control medium or resveratrol (10 μmol/L) for 8 h in the presence or absence of BMSCs. Whole cell lysates from BCWM.1 were subjected to Western blotting using anti-phosphorylated AKT, anti-AKT, anti-phosphorylated ERK, anti-ERK, and anti-α-tubulin.

in combination with other agents active in WM. As part of this study, we cultured BCWM.1 cells for 48 hours with resveratrol (5-10 μmol/L) in the presence or absence of fludarabine (1.25-2.5 μg/mL), dexamethasone (25-50 nmol/L), or bortezomib (5-10 nmol/L). Fludarabine (2.5 μg/mL) induced cytotoxicity in 35% of BCWM.1 cells, which increased to 48% and 70% (data not shown) in the presence of resveratrol at 5 μmol/L (CI, 0.76) and 10 μmol/L (CI, 0.49), respectively, indicating moderate to high synergistic activity (Table 1). Dexamethasone (50 nmol/L) resulted in cytotoxicity in 38% of BCWM.1 cells, which increased to 56% and 78% (data not shown) in the presence of resveratrol at 5 μmol/L (CI, 0.46) and 10 μmol/L (CI, 0.31), respectively, indicating synergism (Table 1). Lastly, we observed that bortezomib (5 nmol/L) induced cytotoxicity in 20% of BCWM.1 cells, which was increased to 42% and 63% (data not shown) in

the presence of resveratrol at 5 μmol/L (CI, 0.90) and 10 μmol/L (CI, 0.79), respectively, indicating low to moderate synergism (Table 1). The fractions affected and the combination index for each of these combinations are summarized in Table 1.

Discussion

The antitumor activity of resveratrol has been shown in several solid tumors including osteosarcoma, esophageal squamous carcinoma, breast cancer, colon cancer, uterine cancer, and hematologic malignancies such as Burkitt's lymphoma, multiple myeloma, and promyelocytic leukemia displaying growth-inhibitory activity, induction of apoptosis, and S-G₂ phase cell cycle arrest. Moreover, in most of the previous studies, it has been reported that resveratrol showed

Table 1. Resveratrol enhances cytotoxicity exerted by fludarabine, dexamethasone, and bortezomib against WM cells

Resveratrol (mmol/L)	Fludarabine (mg/mL)	FA	CI	Effect
5	1.25	0.48	0.76	Moderate synergism
5	2.5	0.57	0.73	Moderate synergism
10	1.25	0.7	0.49	Synergism
10	2.5	0.8	0.36	Synergism
Resveratrol (mmol/L)	Dexamethasone (nmol/L)	FA	CI	Effect
5	25	0.42	0.98	Additive
5	50	0.56	0.46	Synergism
10	25	0.5	0.86	Slight synergism
10	50	0.78	0.31	Synergism
Resveratrol (mmol/L)	Bortezomib (nmol/L)	FA	CI	Effect
5	5	0.42	0.90	Light synergism
5	10	0.63	0.81	Moderate synergism
10	5	0.62	0.79	Moderate synergism
10	10	0.85	0.49	Synergism

NOTE: Fractions affected (FA) and combination indices (CI) of the combinations of resveratrol with fludarabine, dexamethasone, and bortezomib. All experiments were repeated in triplicate. FA for each drug used as single agent: fludarabine, 1.25 mg/mL (0.35); fludarabine, 2.5 mg/mL (0.45); dexamethasone, 25 nmol/L (0.38); dexamethasone, 50 nmol/L (0.43); bortezomib, 5 nmol/L (0.2); bortezomib, 10 nmol/L (0.42); resveratrol, 5 mmol/L (0.14); resveratrol, 10 mmol/L (0.34).

an effective IC_{50} value ranging from 50 to 180 $\mu\text{mol/L}$ (35), and it induced apoptosis only at higher concentrations than those required for resveratrol-induced cytotoxicity and cell cycle arrest, with values ranging from 100 to 200 $\mu\text{mol/L}$ (36). It has been reported that resveratrol seems to be well-tolerated in *in vivo* studies with little toxicity, as shown in mice following the oral administration of a dose as high as 20 mg/kg for 4 weeks (37, 38), which showed significant chemopreventive and antineoplastic activities *in vivo* in several solid tumors (39, 40).

In this study, we show for the first time that resveratrol has significant antitumor activity in WM. Growth inhibition of the WM and IgM-secreting cell lines was observed at IC_{50} values ranging from 10 to 32 $\mu\text{mol/L}$. Furthermore, the proliferation rate of CD19⁺ lymphoplasmacytic cells isolated from patients with relapsed/refractory WM were also significantly reduced by resveratrol treatment. Importantly, resveratrol at 10 to 50 $\mu\text{mol/L}$ had no significant effect on the survival of normal PBMCs. Resveratrol induced apoptosis in WM cells through the activation of both intrinsic and extrinsic apoptotic pathways resulting in caspase-8, caspase-9, caspase-3, and PARP cleavage. Moreover, because the stress-activated protein kinase/JNK pathway facilitates the apoptosis of many chemotherapeutic agents, we investigated this pathway as a determinant of resveratrol-mediated apoptosis in WM. These studies showed that resveratrol triggers JNK phosphorylation. Moreover, the JNK inhibitor SP600125 markedly inhibited resveratrol-induced phosphorylation of JNK and caspase-8 cleavage, suggesting that JNK plays an important role in resveratrol-induced apoptosis in WM cells. In addition, increased apoptosis of BCWM.1 cells correlated with the down-modulation of the antiapoptotic protein Mcl-1, and the induction of the proapoptotic transcription factor p53, both at transcript and protein levels. Importantly, resveratrol treatment was associated with elevated transcript and protein levels of p63 and p73, the members of p53 family with proapoptotic abilities, providing a crucial alternate mechanism of cell growth arrest in the absence of p53, which seems to be frequently mutated in cancers. Furthermore, we showed that resveratrol induced cell cycle arrest, associated with the induction of cyclin-dependent kinase inhibitors, and with the down-regulation of cyclin-dependent kinases, cyclin D1, cyclin D2, and cyclin E1, along with a reduced phosphorylation of Rb.

Importantly, we have shown that resveratrol targets PI3K/AKT, MEK/ERK, and Wnt signaling pathways that act as critical regulators of apoptosis, cell cycle regulation, and tumor proliferation in lymphoproliferative disorders (27–31). Most likely, these same signaling cascades could be affected in normal

cells at higher doses of resveratrol. Previous studies showed that the PI3K/Akt pathway is up-regulated in WM cells as compared with normal controls (41), therefore, this up-regulation in tumor cells could favor a stronger activity of resveratrol at lower doses compared with healthy donor cells. Similarly, it has been shown by gene expression profile analysis that interleukin-6 signaling, which activates both PI3K/Akt and MEK/ERK pathways (19, 42), is up-regulated in WM (43), in which it plays a pivotal role in supporting the growth and survival of neoplastic cells (44). In addition, it has been described as an abnormal overexpression of genes from the Wnt signaling pathway in lymphoproliferative disorders, including B-cell chronic lymphocytic leukemia and multiple myeloma (45). Based on these observations, we could therefore hypothesize that resveratrol-induced down-modulation of those up-regulated pathways could be responsible for a more selective activity of resveratrol against WM cells rather than normal cells.

Because the bone marrow microenvironment plays an important role in the growth and survival of plasma cell malignancies, and blocks chemotherapy-induced apoptosis, we sought to delineate if coculture of WM cells with BMSCs modulated resveratrol-mediated WM cell cytotoxicity. We showed that the adherence of tumor cells to BM stromal cells did not protect against resveratrol-induced WM cytotoxicity. The resveratrol-triggered modulation of signaling pathways induced by the presence of the BM milieu was also investigated. ERK and Akt signaling cascades induced by the adherence of the WM cell line to BM stromal cells were blocked by resveratrol, providing evidence that resveratrol could modulate the signaling changes induced by the bone marrow milieu on WM cells.

Lastly, we showed that resveratrol potentiates the effect of several agents that are active against WM, such as the nucleoside analogue fludarabine (46), the proteasome inhibitor bortezomib (3, 4, 47), and dexamethasone (1). These data may have important implications for future trials, not just in formulating more effective combination therapies with nonoverlapping toxicities, but also in strategies aimed at providing lower doses of agents such as bortezomib, which harbor significant neurotoxicity in WM (3, 4).

In summary, in this study, we have shown that resveratrol induces growth inhibition and apoptosis *in vitro* in WM cells and overcomes the growth-promoting activities of the bone marrow microenvironment. Moreover, the combination of resveratrol with other therapeutic agents mediates synergistic cytotoxicity in WM. Based on these evidences, the present study provides the framework for clinical trials of resveratrol, alone or in combination, in the treatment of WM.

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