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Resveratrol Inhibits Proliferation, Induces Apoptosis and Overcomes Chemoresistance Through Downregulation of STAT3 and Nuclear Factor-κB-Regulated Antiapoptotic and Cell Survival Gene Products in Human Multiple Myeloma Cells

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Running title: Resveratrol inhibits NF-κB and STAT3 in multiple myeloma.

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Abbreviations used: NF-κB, nuclear transcription factor-κB; STAT, signal transducer and activator of transcription; MM, multiple myeloma; EMSA, electrophoretic mobility shift assay; IκBα, inhibitory subunit of NF-κB; IKK, IκBα kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; TRAF2, TNFR-associated factor; IAP, inhibitor-of-apoptosis protein; IL-6, Interleukin 6; COX-2, cyclooxygenase-2; DMBA, dimethylbenz(a)anthracene.

Abstract

Whether resveratrol, a component of red grapes, berries and peanuts, could suppress the proliferation of multiple myeloma (MM) cells by interfering with NF-κB and STAT3 pathways, was investigated. Resveratrol inhibited the proliferation of human multiple myeloma cell lines regardless of whether they were sensitive or resistant to the conventional chemotherapy agents. This stilbene also potentiated the apoptotic effects of Velcade and thalidomide. Resveratrol induced apoptosis as indicated by accumulation of sub G1 population, increase in Bax release and activation of caspase-3. This correlated with downregulation of various proliferative and antiapoptotic gene products including cyclin D1, cIAP-2, XIAP, survivin, Bcl-2, Bcl-xL, Bfl-1 / A1 and TRAF2. In addition, resveratrol downregulated the constitutive activation of AKT. These effects of resveratrol are mediated through suppression of constitutively active NF-κB through inhibition of IκBα kinase; and the phosphorylation of IκBα and of p65. Resveratrol inhibited both the constitutive and the interleukin 6–induced activation of STAT3. When examined CD 138^{+} plasma cells from MM patients, resveratrol inhibited constitutive activation of both NF-κB and STAT3 leading to downregulation of cell proliferation and potentiation of apoptosis induced by Velcade and thalidomide. These mechanistic findings suggest that resveratrol may have a potential in the treatment of multiple myeloma.

Introduction

Multiple myeloma (MM) or plasma cell myeloma is characterized by latent accumulation of secretory plasma cells with a low proliferative index and an extended life span in the bone marrow [1]. The second most prevalent hematologic cancer after non-Hodgkin's lymphoma, multiple myeloma accounts for 10% of all hematologic cancers and approximately 2% of all cancer deaths. Conventional therapy for multiple myeloma involves combinations of vincristine, carmustine (bischloroethylnitrosourea), melphalan, cyclophosphamide, doxorubicin (Adriamycin), and prednisone or dexamethasone [2]. Patients younger than 65 years are usually given high-dose melphalan with autologous stem-cell support, and older patients or those who cannot tolerate such intensive treatment are given standard-dose oral melphalan and dexamethasone. Shortcomings of these treatments are low remission rates (about 5%), short survival times (median 30–36 months) and the development of drug resistance [3,4].

Chemoresistance remains a major therapeutic challenge in MM. The precise mechanism underlying chemoresistance in multiple myeloma is not clear, but one of the main contributors to both chemoresistance and pathogenesis is thought to be activation of NF-κB and STAT3, and dysregulation of apoptosis [4-8]. Overexpression of antiapoptotic molecules has been linked to chemoresistance in MM; in one study, expression of the antiapoptotic protein Bcl-x_L correlated with

chemoresistance, with chemoresponse rates of 83% to 87% among non-Bcl-x_L-expressing cases but only 20% to 31% among Bcl-x_L-expressing cases [9]. Chemoresistance in several types of cancer has been linked to activation of NF-κB, a transcription factor with central roles in the regulation of cell growth, survival, angiogenesis, cell adhesion, and apoptosis [10]. Progression and chemoresistance are also thought to involve interleukin (IL) -6, expression of which is induced by NF-κB, through its regulation of the growth and survival of tumor cells [11,12]. IL-6 leads to constitutive activation of STAT3, which in turn results in expression of high levels of Bcl-x_L [6]. Bcl-2 overexpression, another important characteristic of many multiple myeloma cell lines [13], can rescue cells from glucocorticoid-induced apoptosis [4]. Cell lines resistant to doxorubicin (e.g., RPMI 8226 dox-40) have been shown to overexpress Bcl-x_L [9]. Thus both constitutive activation of NF-κB and STAT3 play an important role in chemoresistance, and inhibition of NF-κB and STAT3 may overcome this chemoresistance.

The use of natural agents may be able to overcome resistance without some of the debilitating side effects of conventional chemotherapy. One such agent is resveratrol, a polyphenol (trans-3, 4', 5-trihydroxystilbene) abundant in red grapes, berries and peanuts. As early as 1997, resveratrol was found to be a potent chemopreventive agent, blocking the initiation, promotion, and progression of tumors induced by the aryl hydrocarbon dimethylbenz(a)anthracene (DMBA) [14]. Since that time, resveratrol has been

shown to inhibit the growth of a wide variety of tumor cells, including lymphoid and myeloid; cancers of breast, prostate, and thyroid; melanoma; head and neck squamous cell carcinoma; and ovarian and cervical carcinoma [15]. Putative mechanisms of growth inhibition include cell cycle arrest, apoptosis, suppression of transcription factors like NF-κB [16,17], and downregulation of various inflammatory gene products such as cyclooxygenase-2 (COX-2) or IL-6 [18]. Our laboratory has also previously reported that resveratrol suppresses DMBA-induced mammary carcinogenesis in rats, through the down-regulation of NF-κB, COX-2, and matrix metalloprotease-9 expression [19].

Whether resveratrol can modulate proliferation of human MM cells or overcome the resistance of such cells to chemotherapy is not known. In exploring these questions, we found that resveratrol can indeed inhibit the proliferation and overcome the chemoresistance of MM cells, and that these effects occur through the suppression of NF-κB and STAT3, which in turn leads to the downregulation of antiapoptotic gene products and increased apoptosis.

Materials and Methods

Reagents and antibodies:

Resveratrol with purity greater than 98% was purchased from Alexis Biochemicals (San Diego, CA). A 20 mM stock solution of resveratrol (molecular weight 228.2) was prepared in ethanol and further diluted in cell culture medium to make working concentrations. Maximum final concentration of ethanol was < 0.1% and was used as a control. Penicillin, streptomycin, RPMI 1640 medium, and fetal calf serum (FCS) were obtained from GIBCO (Grand Island, NY). Glycine, phorbol myristate acetate, lipolysaccharide (LPS), ceramide, bovine serum albumin and mounting medium DPX were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO).

Rabbit polyclonal antibodies to p50, p65, AKT, STAT3, and STAT5 and mouse antibodies against phospho-STAT3 and phospho-STAT5 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against cyclin D1, cIAP2, Bcl-2, Bcl- xL, A1 and TRAF2 were also obtained from Santa Cruz Biotechnology. Anti-IKKα- and anti-IKKβ- antibodies were kindly provided by Imgenex (San Diego, CA). Anti-XIAP antibody was obtained from BD Biosciences; poly (ADP) ribose polymerase (PARP) antibody, from PharMingen (San Diego CA); phospho-IκBα (Ser32) antibody, from New England BioLabs (Beverly, MA); phospho-specific anti-AKT (Ser 473), phospho-specific anti-p65 (serine 529), cleaved caspase-3 from Cell Signaling (Beverly, MA); and goat anti-rabbit-Alexa 594, from Molecular Probes (Eugene, OR). Hoechst 33342,

dexamethasone, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich Chemicals (St. Louis, MO), and γ^{32}P-ATP was from ICN Pharmaceuticals (Costa Mesa, CA). Protein blocking solution (ref no. X0909), Dako cytomation LSAB+System-HRP kit (ref no. K0690), DAB chromogen (DAKO ref. no. K3466) and haematoxylin (DAKO, #S3309) were obtained from Dako cytomation. Velcade (PS-341) was obtained from Millennium (Cambridge, MA). Thalidomide was obtained from Tocris Cookson (St. Louis, MO).

Cell lines and culture conditions:

The human MM cell lines U266 (ATCC TIB-196) and RPMI 8226 (ATCC CCL-155), both plasmacytomas of B cell origin, were obtained from the American Type Culture Collection (Manassas, VA). U266 is known to produce IL-6 and monoclonal antibodies [11,20], RPMI 8226 produces only Ig L chains, with no evidence of H-chain or IL-6 production. RPMI-8226-Dox-6 (a doxorubicin-resistant clone) and RPMI-8226-LR-5 (a melphalan-resistant clone) were kindly provided by Dr. William S. Dalton (H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL). MM.1 (also called MM.1S) cells were established from a peripheral blood sample of a patient with IgA myeloma; these cells secrete L chains, do not express the Epstein-Barr virus genome, and do express the leukocyte antigen DR, plasma cell Ag-1, T9, and T10 antigens [21]. MM.1R, a dexamethasone-resistant variant of MM.1 cells [22], was kindly provided by Dr. Steven T. Rosen of Northwestern University Medical School (Chicago, IL). U266,

RPMI-8226, MM.1, and MM.1R cells, Dox-6 and LR-5 variants were cultured in RPMI 1640 medium containing 1x antibiotic-antimycotic with 10% FCS. Cells were periodically tested by Hoechst staining and by custom PCR for mycoplasma contamination.

Clinical samples:

Marrow samples were obtained from MM patients who gave informed consent and underwent treatment at The University of Texas M. D. Anderson Cancer Center (Houston). Approval was obtained from the U.T. M.D. Anderson Cancer Center institutional review board for these studies. The CD138+ cells were separated as described previously [7]. Table 1 describes the analysis of samples from these patients for various assays.

MTT assay:

The antiproliferative effects of resveratrol on drug-sensitive and drug-resistant cells and patient samples were determined by the MTT dye uptake method as described [23].

Electrophoretic mobility shift assay for NF-κB:

NF-κB activation was analyzed by electrophoretic mobility gel shift assay (EMSA) as described [24].

Immunocytochemistry for NF-κB p65 and STAT3 localization:

Both MM cell lines as well as $CD138^+$ samples from MM patients were examined for NF-κB and for STAT3 by immunocytochemistry method essentially

as described [7,8]. One hundred cells were counted for each patient, and the sample was graded on the basis of a 4-point scale: –, no nuclear positive cells (0%); +, 10-20% cells with nuclear positivity; ++, >50 %with nuclear positivity; +++, more than 80% cells with nuclear positivity.

IKK assay:

The effect of resveratrol on IKK activation was examined by immunecomplex kinase as previously described [25].

Western blotting:

Resveratrol-treated cells were examined by western blot analysis for phospho-IκBα levels in the cytoplasm, phospho-p65 in the nucleus, and for STAT3, STAT5, phospho-STAT3, phospho-STAT5, phospho-AKT, Bax, cIAP-2, XIAP, survivin, Bcl-2, Bcl-xL, Bfl-1/A1 and TRAF2, procaspase-3, cleaved caspase-3 and PARP proteins in the whole-cell extracts as described[26]. The whole-cell extracts were prepared by lysing resveratrol-treated cells in lysis buffer (20 mM Tris [pH 7.4], 250 mM NaCl, 2 mM EDTA (pH 8.0), 0.1% Triton X-100, 0.01 mg/ml aprotinin, 0.005 mg/ml leupeptin, 0.4 mM phenylmethylsulphonylfluoride, and 4 mM sodium orthovanadate). After electrophoresis, the proteins were electrotransferred to a nitrocellulose membrane, blocked with 5% nonfat milk, and probed with various antibodies overnight at 4°C. The blots were then washed, exposed to horseradish peroxidase–conjugated secondary antibodies for 1 h, and finally examined by an ECL reagent (Amersham).

Flow cytometry for cell cycle distribution:

To determine the effect of resveratrol on the cell cycle, U266 and MM1.S cells were first synchronized by serum starvation and then exposed to resveratrol for indicated time intervals. Thereafter cells were washed, fixed with 70% ethanol, and incubated for 30 min at 37°C with 0.1%RNAse A in PBS. Cells were then washed again, resuspended, and stained in PBS containing 25 μg/ml propidium iodide (PI) for 30 min at room temperature. Cell distribution across the cell cycle was analyzed with a FACS Calibur flow cytometer (Becton Dickinson, Bedford, MA).

Live/Dead Assay:

Apoptosis of cells was also determined by Live/Dead assay (Molecular Probes, Eugene, OR, USA) that measures intracellular esterase activity and plasma membrane integrity as described[27].

Annexin V Assay:

One of the early indicators of apoptosis is the rapid translocation and accumulation of the membrane phospholipid phosphatidylserine from the cytoplasmic interface to the extracellular surface. This loss of membrane asymmetry can be detected by utilizing the binding properties of annexin V. This assay was performed as indicated[27].

Statistical analysis:

Statistical analysis was performed by student's unpaired t test. The probability (p) value less than 0.05 were considered statistically significant.

Results

The goal of this study was to determine whether resveratrol can sensitize drug-resistant multiple myeloma cells through the regulation of NF-κB and STAT3 activation. To determine this several MM cell lines and CD138+ cells from MM patients were used.

Resveratrol suppresses the proliferation of drug-resistant MM cell lines and potentiates the apoptotic effect of Velcade and thalidomide:

Resveratrol, at a concentration of 50 μM, suppressed the proliferation of all MM cell types tested, including U266, MM.1R cells (resistant to dexamethasone), RPMI 8226-Dox6 cells (resistant to doxorubicin), and RPMI 8226-LR5 cells (resistant to melphalan) (Figs. 1A through 1D).

Velcade, an inhibitor of proteaosome and thalidomide, an inhibitor of TNF expression, have been approved for the treatment of MM patients. Whether resveratrol can potentiate the effect of these drugs was examined. For this, U266 cells were treated with resveratrol together with either Velcade or thalidomide; and then examined for apoptosis. As shown in Fig. 1E and 1F, resveratrol potentiated the effect of both Velcade and thalidomide. Whether the potentiation by resveratrol was dose-dependent was examined. For this cells were treated with different concentrations of resveratrol together with different concentration of either Velcade or thalidomide. As can be seen in Fig. 1G, potentiation can be seen at both 25 and 35 μM of resveratrol. To further confirm the potentiation effect of resveratrol, we used annexin V staining which detects an early stage of

apoptosis. These results also indicated enhancement of apoptotic effects of Velcade (Fig. 1H, left panel) and thalidomide (Fig. 1H, right panel) by resveratrol.

Resveratrol causes accumulation of MM cells in sub G1 phase, increases release of Bax protein accumulation and activates caspase-3:

To further confirm that resveratrol inhibits proliferation of MM cells through induction of cell cycle arrest, we analyzed cell cycle distribution after PI staining. We found that resveratrol caused statistically significant accumulation of cell population in sub G1 phase after the treatment of U266 (Fig. 2A) and MM1.S cells (Figs. 2B) with resveratrol for 12h and 24 h. Increased accumulation of pro-apoptotic protein Bax, was also seen in time-dependent manner on treatment of U-266 cells with resveratrol (Fig. 2C). Cleavage of procaspase-3 to caspase-3, and caspase-3-mediated cleavage of PARP, are other characteristic feature of apoptosis, that were induced by resveratrol in U266 (Fig. 2D) and in MM1.S cells (Fig 2E). Thus these results suggest that resveratrol induces apoptosis through the activation of caspases.

Resveratrol suppresses AKT activation and inhibits the expression of antiapoptotic proteins in MM cells:

Activation of AKT also plays a major role in cell survival [28]. Whether resveratrol modulates the activation of AKT in MM cells, was therefore investigated. AKT was found to be constitutively active in U266 cells, and resveratrol was found to suppress constitutively phosphorylated AKT levels in a time-dependent manner

(Fig. 3A), indicating that these reduced AKT levels may contribute towards increasing the apoptosis of MM cells. Whether the expression of genes implicated in tumor cell proliferation (cyclin D1), and survival (cIAP-2, XIAP, survivin, Bcl-2, Bcl-xL, Bfl-1 / A1, and TRAF2) are regulated by resveratrol was investigated. We found that resveratrol downregulated the constitutive expression of cyclin D1 in three different MM cell lines including U266 (Fig. 3B, left panel), MM1.S (Fig. 3B, middle panel) and RPMI 8826 (Fig. 3B, right panel) cells. Also resveratrol treatment decreased the levels of antiapoptotic gene products cIAP-2, XIAP, survivin, Bcl-2, Bcl-xL, Bfl-1 / A1 and TRAF2 proteins in U266 (Fig. 3C left panel), XIAP and Bcl-xL (Fig. 3C middle panel) in MM1.S, and (Fig. 3C right panel) in RPMI 8826 cells.

Resveratrol inhibits constitutively active NF-κB in MM cells:

Because the expression of cyclin D1, cIAP-2, XIAP, survivin, Bcl-2, Bcl-xL, Bfl-1 / A1, and TRAF2 is regulated by NF-κB[29], whether suppression of expression of these gene products by resveratrol is through the downregulation of NF-κB, was examined. By using EMSA, We found that treatment of MM cells with resveratrol suppressed the constitutive active NF-κB in a dose and time-dependent manner in U266 (Fig. 4A), in MM1.S (Fig. 4B), and in RPMI 8826 (Fig. 4C) cells.

To show that the inhibited band visualized by EMSA in U266 cells was indeed NF-κB, we incubated the nuclear extracts from these cells with antibodies against p50 or p65 subunits and performed the EMSA. Antibodies to either

subunit of NF-κB shifted the band to one of higher molecular weight (Fig. 4D), suggesting that the constitutively activated complex consisted of p50 and p65 subunits. A 100-fold excess of cold oligo nearly eradicated the band but incubation with mutated oligo failed to compete, further confirming the specificity of NF-κB binding.

To determine whether the inhibition of NF-κB by resveratrol resulted from inhibition of IKK, U266 cells were exposed to resveratrol for various times, after which cells were lysed and IKK complexes were extracted by immunoprecipitating with IKK-α antibody, and subjected to an immunocomplex kinase assay with GST-IκBα used as a substrate. Resveratrol inhibited IKK activity without affecting the levels of IKK-α and IKK-β proteins (Fig. 4E). Resveratrol also suppressed the phosphorylation of both IκBα (Fig. 4F) and of p65 (Figs. 4G). Whether resveratrol suppreses the p65 nuclear localization, was examined by immunocytochemistry after 24-h incubation with 50 μM resveratrol. The results show that resveratrol inhibited the appearance of p65 in the nucleus (Fig. 4H).

Resveratrol inhibits constitutively active and IL-6 inducible STAT3 activation in MM cells:

Because the expression of cyclin D1 and bcl-xl is also regulated by STAT3[30, 31], whether resveratrol also modulates STAT3 activation was investigated. For this, we exposed to U266 cells with various doses of resveratrol and for various times and assessed the levels of phosphorylated STAT3 by western blotting. STAT3

was found to be constitutively active in these cells and resveratrol downregulated phospho-STAT3 levels in a time- and dose-dependent manner (Figs. 5A, and 5B). This treatment did not change the expression of phosphorylated STAT5 levels (Fig 5C), indicating that the effect of resveratrol is specific to STAT3.

Phosphorylation of STAT3 induces its dimerization and translocation from the cytoplasm into the nucleus [32]. To confirm that resveratrol suppresses nuclear translocation of STAT3, we stained resveratrol-treated and untreated cells with anti-STAT3 antibody and found that exposure to resveratrol substantially inhibited the translocation of STAT3 from the cytoplasm to the nucleus (Fig. 5D).

Because IL-6 is known to activate STAT3, we tested whether resveratrol would affect STAT3 activation induced by IL-6. We confirmed that IL-6 induced phospho-STAT3 in RPMI 8266 cells as early as 5 min after exposure and that this induction increased further over time (Fig. 5E). We also found that treatment with resveratrol led to suppression of IL-6–induced phosphorylation of STAT3, an effect that also increased over time (Fig. 5F). These results suggest that resveratrol downregulates both constitutive and inducible STAT3 activation.

Resveratrol potentiates the effect of Velcade and Thalidomide on NF-κB and STAT3 activation in MM cells:

Our results indicate that resveratrol can potentiate the apoptotic effects of Velcade and thalidomide. Whether this correlates with downregulation of NF-κB and STAT3 activation was examined. To investigate this, U266 cells were

exposed to sub optimal doses of resveratrol, Velcade and thalidomide; and then examined for NF-κB and STAT3. As shown in Fig. 6, resveratrol potentiated the effect of Velcade and thalidomide on NF-κB (Fig. 6A and 6B) and on STAT3 (Fig. 6C and 6D).

Resveratrol inhibits the proliferation of CD138+ cells from MM patients and downregulates NF-κB and STAT3 activation:

Till now all the studies were carried with MM cell lines. We also investigated the effect of resveratrol on proliferation of CD138+ cells from six MM patients as described in Table 1. Cells were exposed to different concentrations of resveratrol and then examined for cell proliferation by the MTT method. As shown in Fig. 7A, exposure of CD138+ cells from all six MM patients to resveratrol decreased cell proliferation in a dose-dependent manner, except patient number 4 where maximum inhibition was observed at 10 μM of resveratrol concentration and not beyond.

Whether resveratrol-induced inhibition of proliferation of CD138+ cells from MM patients, leads to the down-regulation of NF-κB and STAT3 activation, was investigated. We found that resveratrol was able to inhibit constitutively active NF-κB (Fig. 7B) and nuclear translocation of STAT3 (Fig. 7C) in CD138+ cells from MM patients in a statistically significant manner ($p<0.05$).

Resveratrol potentiates the apoptotic effect of Velcade and thalidomide in CD138+ cells from MM patients:

Whether resveratrol potentiates the apoptotic effect of Velcade and thalidomide in CD138+ cells from MM patients was also investigated. Using annexin V staining (which detects an early stage of apoptosis), we found that resveratrol enhances the apoptotic effect of Velcade and thalidomide in CD138+ cells derived from MM patients #8 and #9 (Fig 7D).

Discussion

The aim of this study was to determine whether resveratrol could suppress the proliferation of multiple myeloma (MM) cells by interfering with NF-κB and STAT3 pathways. Resveratrol inhibited the proliferation of human multiple myeloma cell lines regardless of whether they were sensitive or resistant to the conventional chemotherapeutic agents. It also potentiated the apoptotic effects of Velcade and thalidomide. Resveratrol induced sub G1 accumulation, increased Bax leading to caspase-3 activation. This correlated with downregulation of various proliferative and antiapoptotic gene products including cyclin D1, cIAP-2, XIAP, survivin, Bcl-2, Bcl-xL, Bfl-1 / A1 and TRAF2. These effects of resveratrol are mediated through suppression of constitutively active NF-κB through inhibition of IκBα kinase. Resveratrol also inhibited both the constitutive and the interleukin 6–induced activation of STAT3. We found that resveratrol inhibited the survival of CD 138^{+} plasma cells from MM patients, potentiated the apoptotic effect of Velcade and thalidomide, and this correlated with suppression of constitutive activation of both NF-κB and STAT3.

In agreement with previous reports [4,5,33], we found that all MM cell lines expressed constitutively activated NF-κB, and that resveratrol suppressed the activation. Although resveratrol has been shown to inhibit inducible NF-κB activation in cell lines of various origins [18], whether resveratrol can also inhibit constitutively activated NF-κB in MM cell lines has not been previously reported. Resveratrol inhibits NF-κB through suppression of constitutively active IKK,

which is needed for NF-κB activation. We found that inhibition of IKK by resveratrol led to inhibition of phosphorylation of both IκBα and p65; we also found that resveratrol suppressed constitutively active AKT. Both AKT and IKK have been shown to phosphorylate p65 [34-36]. AKT has been shown to provide survival signals and inhibit apoptosis [28, 37].

In addition to NF-κB, we also found for first time that resveratrol could suppress both constitutive and inducible STAT3 activation in multiple myeloma cells and that these effects were specific to STAT3, as resveratrol had no effect on STAT5 phosphorylation. STAT3 phosphorylation plays a critical role in transformation and proliferation of tumor cells. All Src-transformed cell lines have persistently activated STAT3, and dominant-negative STAT3 blocks transformation [38,39]. Dominant-negative STAT3 has also been shown to induce apoptosis in cells with constitutively active STAT3 [6]. Other forms of cancer, including head and neck cancers [40], hepatocellular carcinoma [41], lymphomas, and leukemia [42] also have constitutively active STAT3. The suppression of constitutive active STAT3 in MM cells by resveratrol raises the possibility that this novel STAT3 inhibitor might also inhibit the constitutively activated STAT3 in other types of cancer cells.

We also found that resveratrol suppressed several genes that are regulated by NF-κB and STAT3; including the proliferative (cyclin D1) and antiapoptotic gene products (cIAP-2, XIAP, survivin, Bcl-2, Bcl-xL, Bfl-1 / A1 and TRAF2[33]). Constitutively active STAT3 can contribute to oncogenesis by protecting cancer

cells from apoptosis; this implies that suppression of STAT3 activation by agents such as resveratrol could facilitate apoptosis. Constitutively active STAT3 has been implicated in the induction of resistance to apoptosis [6], possibly through the expression of Bcl-2 and cyclin D1 [43,44]. Expression of Bcl-xL is known to be regulated by both STAT3 [45] and NF-κB [46] and to be overexpressed in MM cells [9]. Bcl-xL can block cell death induced by variety of chemotherapeutic agents [47], and expression of Bcl-xL has been correlated with chemoresistance in patients with MM[9]. Our results confirm that resveratrol downregulated the expression of Bcl-2 and Bcl-xL in MM cells, which may be responsible for the decline in viability of those cells.

To our knowledge this is the first report of the ability of resveratrol to overcome chemotherapy-induced resistance in MM cells. Resveratrol-induced cell death in MM.1R cells (resistant to dexamethasone), RPMI 8226-Dox6-R cells (resistant to doxorubicin) and RPMI 8226-LR5-R cells (resistant to melphalan) was comparable to that in their drug-sensitive counterparts. Resveratrol has been reported to enhance paclitaxel-induced cell death in RPMI 8226 cells [48], but the mechanism by which this takes place is unknown. Because paclitaxel activates NF-κB [49], it is possible that resveratrol sensitizes cells to paclitaxel by downregulating NF-κB.

Recently, a proteasome inhibitor (PS341 also called Velcade) and a TNF inhibitor (Thalidomide) were approved for the treatment of multiple myeloma [50,51]. Both of these inhibitors also suppress NF-κB activation [52,53] but both have

severe side effects. We also found for the first time that resveratrol potentiates the apoptotic effect of Velcade and thalidomide in CD 138^{+} plasma cells from MM patients. However, the variation in sensitivity of different patient samples to resveratrol or to the combination of drugs could be due to regulation of apoptosis by multiple mechanisms in cells from patients; and that these mechanisms may vary from patient to patient.

Resveratrol has been shown to be well tolerated in animal studies, with little toxicity [14,18,54]. As high as 3000 mg resveratrol / kg body weight / day for 4 weeks was found to have no side-effects in rats [55]. We contend that the apparent pharmacologic safety of resveratrol and its ability to downregulate the expression of several genes involved in cell survival and chemoresistance [18] provides a sufficient rationale for testing resveratrol in patients with MM.

MM that has relapsed after conventional-dose therapy or stem-cell transplantation is typically treated with high-dose corticosteroids, thalidomide, or velcade. However, disease in significant proportions of patients does not respond to these agents. Moreover, prolonged exposure leads to the development of resistance and toxicity, and progression-free and overall survival times are short. Collectively, safety information from preclinical studies and the ability of resveratrol to suppress NF-κB and STAT-3 activation, inhibit IL-6 signaling, downregulate the expression of cyclin D1, and Bcl-xL, inhibit cell proliferation, ability to potentiate the effect of Velcade and thalidomide, and to overcome drug resistance provide a sound basis for conducting clinical trials

with resveratrol, alone or in combination with other agents, to enhance treatment efficacy, reduce toxicity, and overcome chemoresistance of relapsed or refractory MM.

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Figure Legends

Figure 1. Resveratrol suppresses the proliferation of drug-resistant MM cell lines and potentiates the apoptotic effect of Velcade and thalidomide. For panels A, B, C & D, U266 cells (5 x 10^3 / 100 µl, panel A); dexamethasone-sensitive (panel B, left) and dexamethasone-resistant (panel B, right) MM.1 cells (20 x 10^3 / 100 µl); doxorubicin-sensitive (panel C, left) and doxorubicin-resistant (panel C, right) RPMI 8266 cells (20 x 10^3 / 100 µl); and melphalan-sensitive (panel D, left) and melphalan-resistant (panel D, right) RPMI 8266 cells were plated in triplicate, treated with 50 µM resveratrol, and then subjected to MTT assay on days 2, 4, or 6 to analyze proliferation of cells. Hollow circle represents control and filled circle represents resveratrol treated cells. Each point on line is an average of triplicate value. Resveratrol induced inhibition of cell growth at days 2 and 4 was statistically significant ($p < 0.05$). For panels E and F, U266 cells (1×10^6 / ml) were treated with 25 µM resveratrol, and 20nM Velcade (Fig. 1E) or 10 µg / ml Thalidomide (Fig 1F) alone or in combination for 24 h at 37°C. Cells were stained with a Live / Dead assay reagent for 30 min and then analyzed under a fluorescence microscope as described in Materials and Methods. Percent apoptosis is indicated in the inset. For panel G, U266 cells (1×10^6 / ml) were treated with indicated concentrations of resveratrol, velcade and thalidomide alone or in combination for 24 h at 37°C. Cells were stained with a Live / Dead assay reagent for 30 min and then analyzed under a fluorescence microscope as described in Materials and Methods. The results shown are % apoptosis and are

representative of three independent experiments. Standard deviations between the triplicates are indicated.

For panel H, U266 cells (1×10^6/ml) were treated with 25 μM resveratrol, 20nM Velcade (left panel) or 10 μg/ml thalidomide (right panel) alone or in combination for 24 h at 37°C. Cells were incubated with anti-annexin V antibody conjugated with FITC and then analyzed with a flow cytometer for early apoptotic effects. The results shown are representative of three independent experiments. * represents that values are significantly ($p<0.05$) different than control as well as single agent.

Figure 2. Resveratrol causes accumulation of MM cells in sub G1 phase, increases release of Bax protein accumulation and activates caspase-3.

For panel A, U266 cells (2×10^6/mL) were synchronized by incubation overnight in the absence of serum and then treated with 50 μM resveratrol for 0, 12, or 24 h, after which the cells were washed, fixed, stained with PI, and analyzed for DNA content by flow cytometry. Results typical of 3 independent experiments are shown. * indicates that p value is <0.05. For panel B, MM1.S cells (2×10^6/mL) were synchronized by incubation overnight in the absence of serum and then treated with 50 μM resveratrol for 0, 12, or 24 h, after which the cells were washed, fixed, stained with PI, and analyzed for DNA content by flow cytometry. Results typical of 3 independent experiments are shown. * indicates that p value is <0.05. For panel C, U266 cells (2×10^6/ml) were treated with 50 μM resveratrol for the indicated times and whole-cell extracts were prepared,

separated on SDS-PAGE, and subjected to western blot using antibody against Bax. The same blot were stripped and re-probed with β-actin antibody to show equal protein loading. For panel D, U266 cells were treated as described in (C) and whole-cell extracts were prepared, separated on SDS-PAGE, and subjected to western blot using antibodies against indicated proteins. The same blots were stripped and re-probed with β-actin antibody to show equal protein loading. For panel E, MM1.S cells were treated as described in (C) and whole-cell extracts were prepared, separated on SDS-PAGE, and subjected to western blot using antibody against PARP. The same blots were stripped and re-probed with β-actin antibody to show equal protein loading. The results shown are representative of three independent experiments.

Figure 3. Resveratrol suppresses AKT activation and inhibits the expression of antiapoptotic proteins in MM cells.

For panel A, U266 cells (2×10^6/ml) were treated with 50 μM resveratrol for the indicated times and whole-cell extracts were prepared, separated on SDS-PAGE, and subjected to western blotting with a phospho-AKT antibody. The same blot were stripped and re-probed with AKT antibody to show equal protein loading. For panel B, U266 cells (left panel)/MM1.S (middle panel)/RPMI 8826 (right panel) were treated with 50 μM resveratrol for the indicated times and whole-cell extract were prepared, separated on SDS-PAGE, and subjected to western blot analysis using cyclin D1 antibody. The same blot was stripped and re-probed

with β-actin antibody to verify equal protein loading. For panel C, U266 / MM1.S / RPMI 8826 cells were treated as described in (A) and whole-cell extracts were prepared, separated on SDS-PAGE, and subjected to western blot using antibodies against indicated proteins. The same blots were stripped and re-probed with β-actin antibody to show equal protein loading. The results shown are representative of three independent experiments.

Figure 4. Resveratrol inhibits constitutively active NF-κB in MM cells.

Resveratrol suppressed NF-κB in a time-dependent and a dose-dependent manner (panel A) in U266, (panel B) in MM1.S and, (panel C) RPMI 8826 cells. For panel A (left), U266 cells (2×10^6 / ml) were treated with 50 μM resveratrol for 0, 3,6,12 and 24 h; and then tested for NF-κB by EMSA. For panel A (right), U266 cells (2×10^6 / ml) were treated with the indicated concentrations of resveratrol for 24 hours and then tested for NF-κB by EMSA. (B, left panel) MM1.S cells (2×10^6 / ml) were treated with 50 μM resveratrol for 0, 3,6,12 and 24h; and then tested for NF-κB by EMSA. (B, right panel) MM1.S cells (2×10^6 / ml) were treated with the indicated concentrations of resveratrol for 24 hours and then tested for NF-κB by EMSA. For panel C (left) RPMI 8826 cells (2×10^6 / ml) were treated with 50 μM resveratrol for 0, 3,6,12 and 24h; and then tested for NF-κB by EMSA. For panel C (right), RPMI 8826 cells (2×10^6 / ml) were treated with the indicated concentrations of resveratrol for 24 hours and then tested for NF-κB by EMSA. For panel D, NF-κB DNA binding was specific and the activated complex

consisted of p50 and p65 subunits. The results shown are representative of three independent experiments. For panel E, To measure IKK activity, (4×10^6 / ml) were incubated with 50 μM resveratrol for the indicated times, after which whole cell lysates were prepared and immunoprecipitated with an antibody against IKK-α and analyzed with an immunocomplex kinase assay. (Bottom) IKK protein levels were assessed by fractionating whole cell extracts on SDS-PAGE and examining them with western blot using anti-IKK-α and anti-IKK-β antibodies. For panel F and G, To assess phosphorylation of IκBα (F) and p65 (G), U266 cells (2×10^6 / ml) were treated with 50 μM resveratrol for indicated times and subjected to cytoplasmic fractionation. Then, 30-μg extracts were resolved on 7.5% SDS-PAGE gel and electrotransferred onto nitrocellulose membranes. The cytoplasmic fraction was probed for phospho-IκBα (F), and the nuclear fraction was probed for phospho-p65 (G). The same blots were stripped and reprobed with β-actin antibody to verify equal protein loading. For panel H, Resveratrol induced translocation of activated nuclear p65 from the nucleus to the cytoplasm. U266 cells (2×10^6 / ml) were incubated with medium (left) or with 50 μM resveratrol (right) for 24 hours and then analyzed for the intracellular distribution of p65 by immunocytochemistry. Red indicates p65, and blue indicates nuclei (original magnification ×200). The results shown are representative of three independent experiments.

Figure 5. Resveratrol inhibits constitutively active and IL-6 inducible STAT3 activation in MM cells.

Resveratrol suppressed phospho-STAT3 levels in a time-dependent (A) and dose-dependent (B) manner. For panel A, U266 cells (2×10^6 / ml) were treated with the 50 μM resveratrol for the indicated times, after which whole-cell extracts were prepared and 30-μg portions of those extracts were resolved on 7.5% SDS-PAGE gel, electrotransferred onto nitrocellulose membranes, and probed for phospho-STAT3. The same blots were stripped and reprobed with STAT3 antibody to verify equal protein loading. For panel B, U266 cells (2×10^6 / ml) were treated with the indicated concentration of resveratrol 3 h, after which western blotting was performed as described for panel A. The same blots were stripped and re-probed with STAT3 antibody to verify equal protein loading. For panel C, Resveratrol had no effect on STAT5 or phospho-STAT5 protein levels. U266 cells (2×10^6 / ml) were treated with 50 μM resveratrol for the indicated times. Whole cell extracts were prepared, fractionated on SDS-PAGE and examined by western blot using antibodies against phospho-STAT5 and STAT5. For panel D, Resveratrol causes inhibition of translocation of STAT3 to the nucleus. U266 cells (1×10^5 / ml) were incubated with or without 50 μM resveratrol for 3 h and then analyzed for the intracelullar distribution of STAT3 by immunocytochemistry. Red indicates STAT3, and blue indicates nucleus (original magnification ×200). For panel E and F, Resveratrol downregulates IL-6–induced phospho-STAT3. (E) RPMI 8226 cells (2×10^6 / mL) were treated with

IL-6 (10 ng / ml) for indicated times, whole cell extracts were prepared, and phospho-STAT3 was detected by western blot as described in Materials and Methods. The same blots were stripped and reprobed with STAT3 antibody to verify equal protein loading. (F) RPMI 8226 cells (2×10^6 / ml) were treated with 50 μM resveratrol for the indicated times and then stimulated with IL-6 (10 ng / ml) for 15 minutes. Whole-cell extracts were then prepared and analyzed for phospho-STAT3 by western blotting. The same blots were stripped and re-probed with STAT3 antibody to verify equal protein loading. The results shown are representative of three independent experiments.

Figure 6. Resveratrol potentiates the effect of Velcade and Thalidomide on NF-κB and STAT3 activation in MM cells.

For panel A, U266 cells (1×10^6 / ml) were treated with 25 μM resveratrol or 10nM velcade alone or in combination for 24 h at 37°C and then tested for NF-κB by EMSA. For panel B, U266 cells (1×10^6 / ml) were treated with 25 μM resveratrol, or 10 μg / ml thalidomide alone or in combination for 24 h at 37°C and then tested for NF-κB by EMSA. The results shown are representative of three independent experiments. For panel C, U266 cells (1×10^6 / ml) were treated with 25 μM resveratrol or 10nM velcade alone or in combination for 3 h at 37°C Whole-cell extracts were prepared and 30-μg portions of those extracts were resolved on 7.5% SDS-PAGE gel, electrotransferred onto nitrocellulose membranes, and probed for antibody against phospho-STAT3. The same blots

were stripped and reprobed with STAT3 antibody to verify equal protein loading. For panel D, U266 cells (1×10^6 / ml) were treated with 25 μM resveratrol, or 10 μg / ml thalidomide alone or in combination for 3 h at 37°C after which western blotting was performed as described for panel C. The results shown are representative of three independent experiments.

Figure 7. Resveratrol inhibits the proliferation of CD138+ cells from MM patients and downregulates NF-κB and STAT3 activation.

For panel A, enriched CD138+ cells (2×10^5 / 0.1 mL) from bone marrow aspirates of multiple myeloma patients were cultured in the absence or presence of indicated concentrations of resveratrol for 24 hours, and cell proliferation was measured by MTT assay as described in "Patients, materials, and methods." Values represent the mean ± SD of triplicate cultures. * indicates that p value is <0.05. For panel B, enriched CD138+ cells (2×10^6 cells) from bone marrow aspirates of MM patients as indicated were cultured in absence or presence of resveratrol, (50 μM) for 12 hours and then tested for NF-κB activity in the nuclei by electrophoretic mobility shift assay as described in Materials and Methods. For panel C, STAT3 activation status was determined by fixing the untreated and 12 h resveratrol treated (50 μM, 100 μM) enriched CD138+ cells (2×10^6 cells) on slides by cytospin followed by immunocytochemistry for STAT3 as described in methods section. Red stain indicates the specific nuclear positivity for STAT3, whereas blue counterstain indicates the nuclei, which are negative for STAT3.

Patient's numbers are indicated beside each panel. Original magnification, x 200. One hundred cells were counted for each patient. Grading: +, less than 10% cells with nuclear positivity; ++, 10% to 50% cells with nuclear positivity; +++, more than 51% cells with nuclear positivity. ++ and + indicate significantly different nuclear ($p<0.05$) positivity than untreated cells. For panel D, CD138+ cells (2×10^4 cells) from patients #8 and #9 were treated with 25 μM resveratrol, 20nM Velcade, 10 μg / ml thalidomide either alone or in combination for 24 h at 37°C. Cells were incubated with anti-annexin V antibody conjugated with FITC and then analyzed by a flow cytometer for early apoptotic effects.

Table 1

Analysis of CD138+ samples derived from multiple myeloma patients for proliferation, apoptosis, STAT3 and NF-kB

Patient #	MTT	STAT3	NF-κB	Annexin
1		+		
2	+	+		
3	+		+	
4	+		+	
5	+	+	+	
6	+	+	+	
7	+	+		
8				+
9				+

Proliferation was measured by the MTT method and apoptosis was measured by Annexin method.

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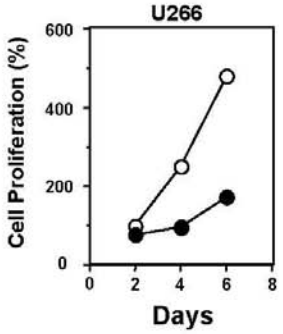
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4
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8
Days

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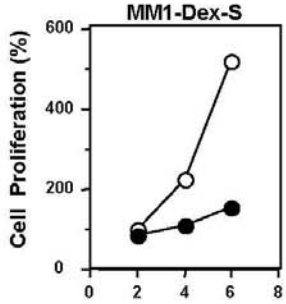
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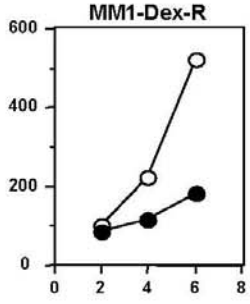
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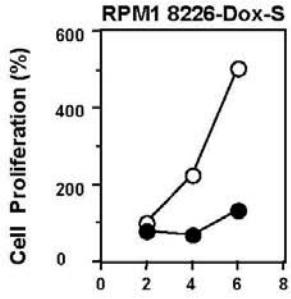
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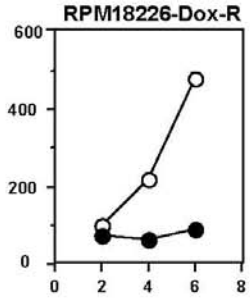
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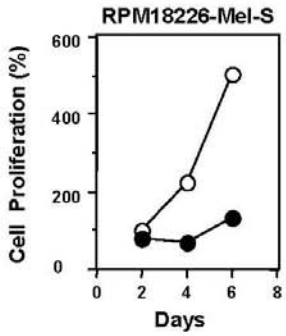
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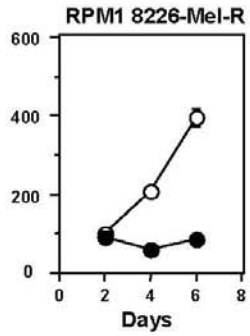
Days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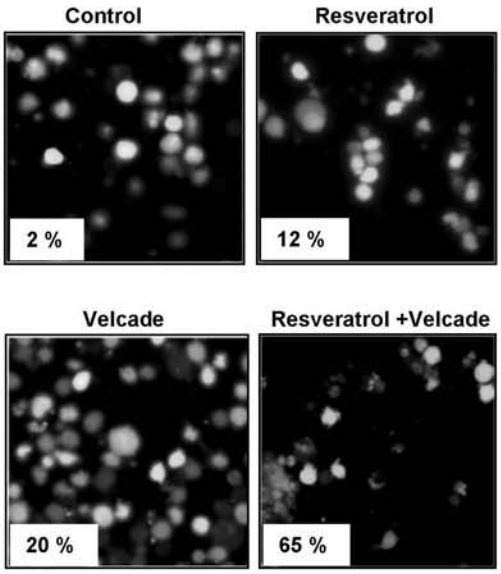

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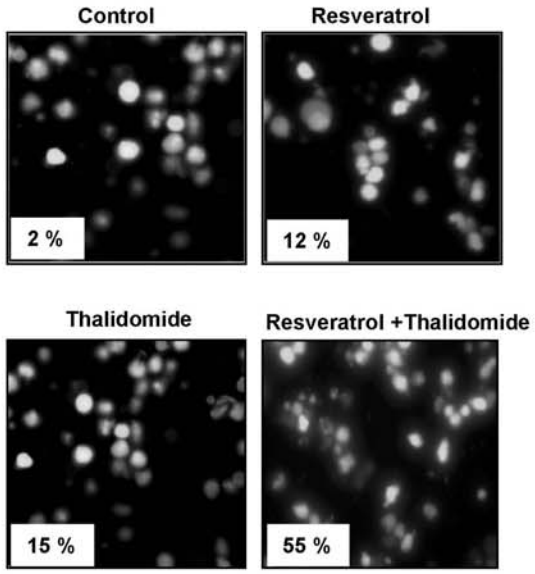
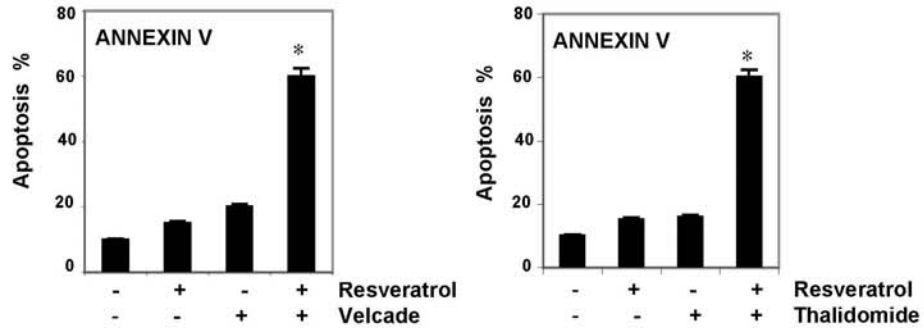


G.

Resveratrol (μM)	0	Velcade (nM)			Thalodimide (μg/ml)		
		10	20	30	5	10	20
0	1±0.57	5±0.5	20±1.63	45±2.45	5±0.82	15±3.32	35±2.45
15	6±0.82	12±0.96	25±1.15	45±8.54	10±1.63	20±2.99	35±6.45
25	12±2.06	25±3.56	65±4.08	92±5.06	30±1.15	55±4.08	85±4.08
35	40±2.98	60±6.45	98±1.15	100±0.5	45±4.08	90±3.09	100±0.5

H.



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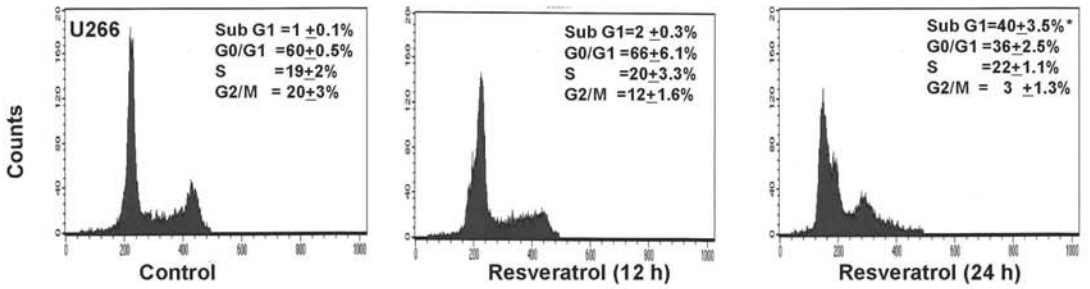
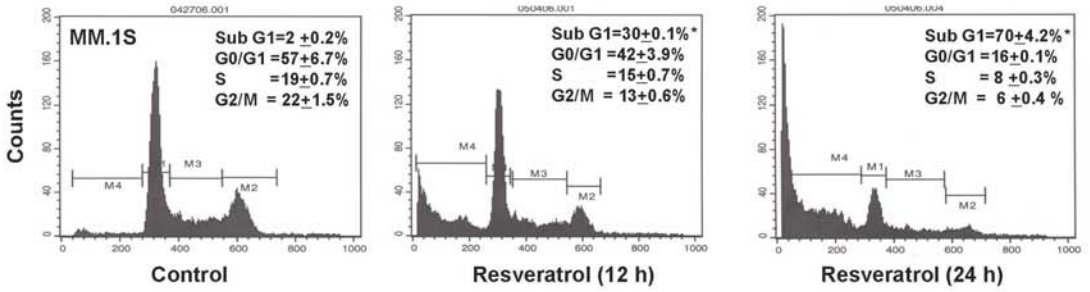
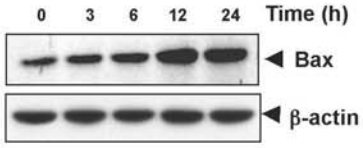

U266
Sub G1 =1 ±0.1%
G0/G1 =60±0.5%
S =19±2%
G2/M = 20±3%
Sub G1=2 ±0.3%
G0/G1 =66±6.1%
S =20±3.3%
G2/M =12±1.6%
Sub G1=40±3.5%*
G0/G1 =36±2.5%
S =22±1.1%
G2/M = 3 ±1.3%
Counts
Control
Resveratrol (12 h)
Resveratrol (24 h)

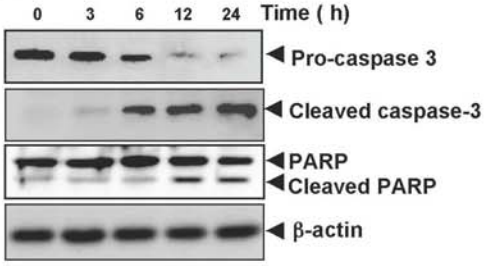
B.


MM.1S
Sub G1=2 ±0.2%
G0/G1 =57±6.7%
S =19±0.7%
G2/M = 22±1.5%
Sub G1=30±0.1%*
G0/G1 =42±3.9%
S =15±0.7%
G2/M = 13±0.6%
Sub G1=70±4.2%*
G0/G1 =16±0.1%
S = 8 ±0.3%
G2/M = 6 ±0.4 %
M1
M2
M3
M4
Counts
Control
Resveratrol (12 h)
Resveratrol (24 h)

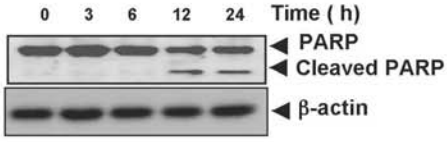
C.


0 3 6 12 24 Time (h)
Bax
β-actin

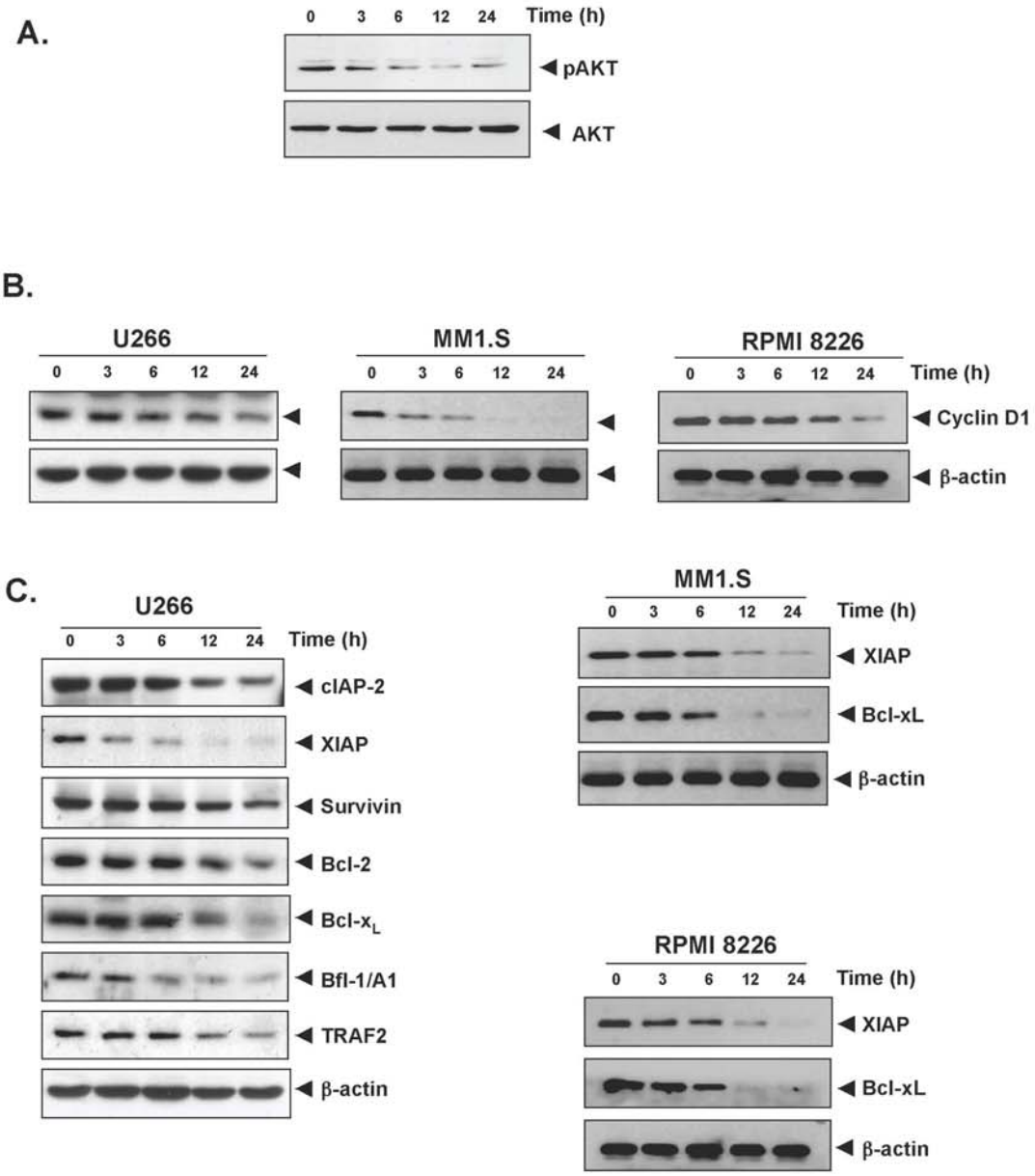
D.

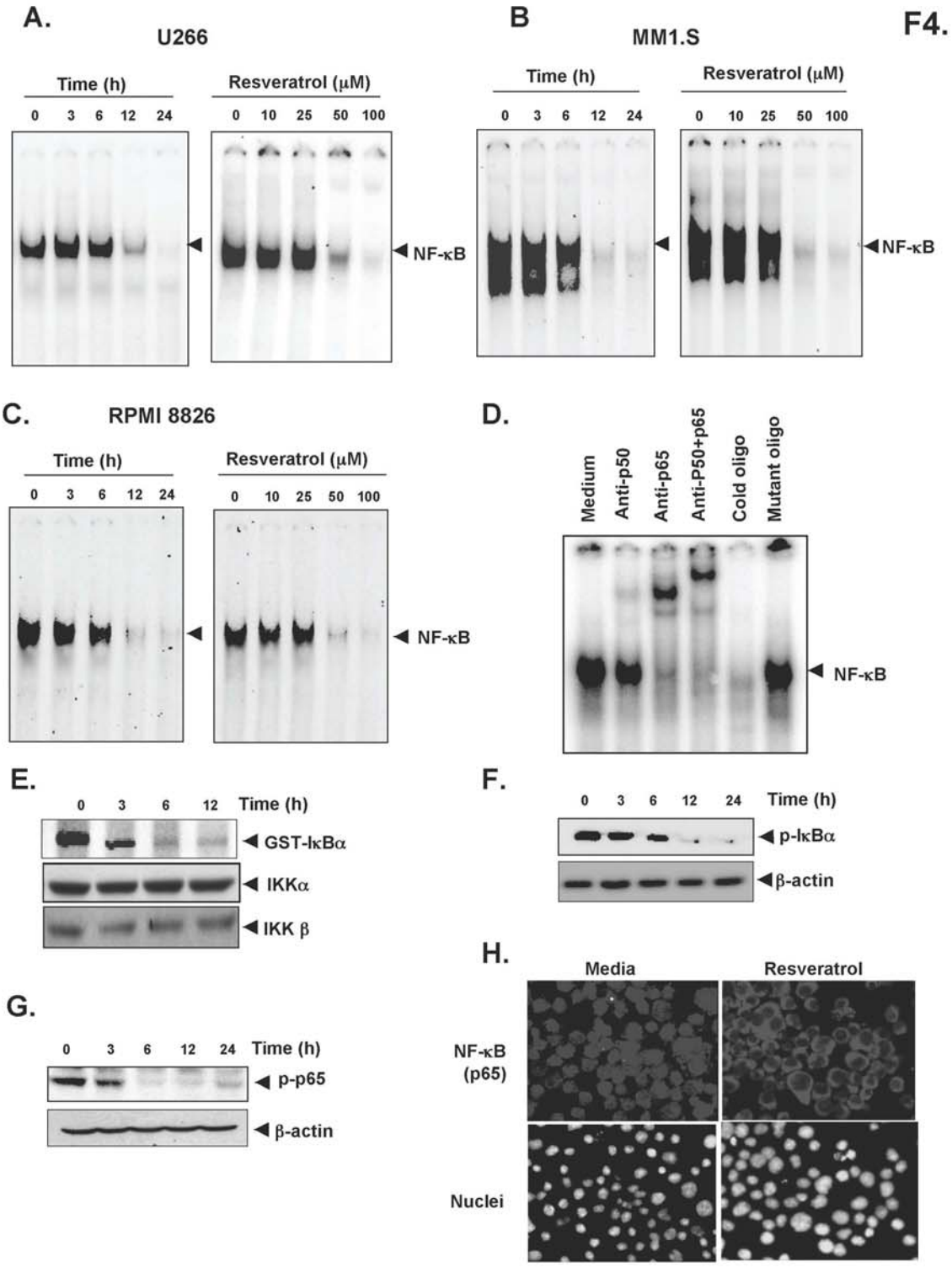

0 3 6 12 24 Time (h)
Pro-caspase 3
Cleaved caspase-3
PARP
Cleaved PARP
β-actin

E.


0 3 6 12 24 Time (h)
PARP
Cleaved PARP
β-actin

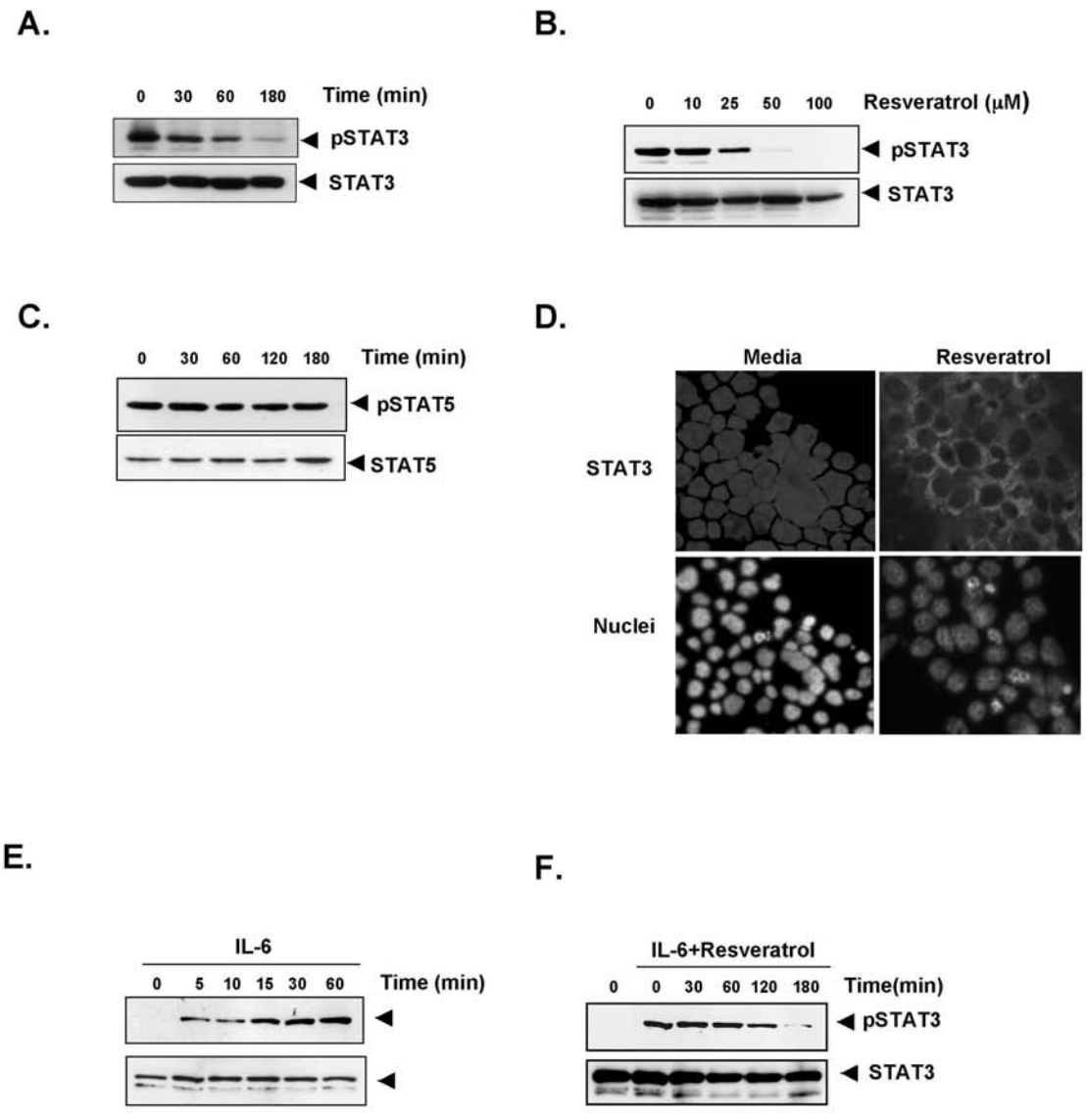
F3.


A.
0 3 6 12 24 Time (h)
pAKT
AKT
B.
U266
MM1.S
RPMI 8226
0 3 6 12 24 Time (h)
Cyclin D1
β-actin
C.
U266
0 3 6 12 24 Time (h)
cIAP-2
XIAP
Survivin
Bcl-2
Bcl-xL
Bfl-1/A1
TRAF2
β-actin
MM1.S
0 3 6 12 24 Time (h)
XIAP
Bcl-xL
β-actin
RPMI 8226
0 3 6 12 24 Time (h)
XIAP
Bcl-xL
β-actin

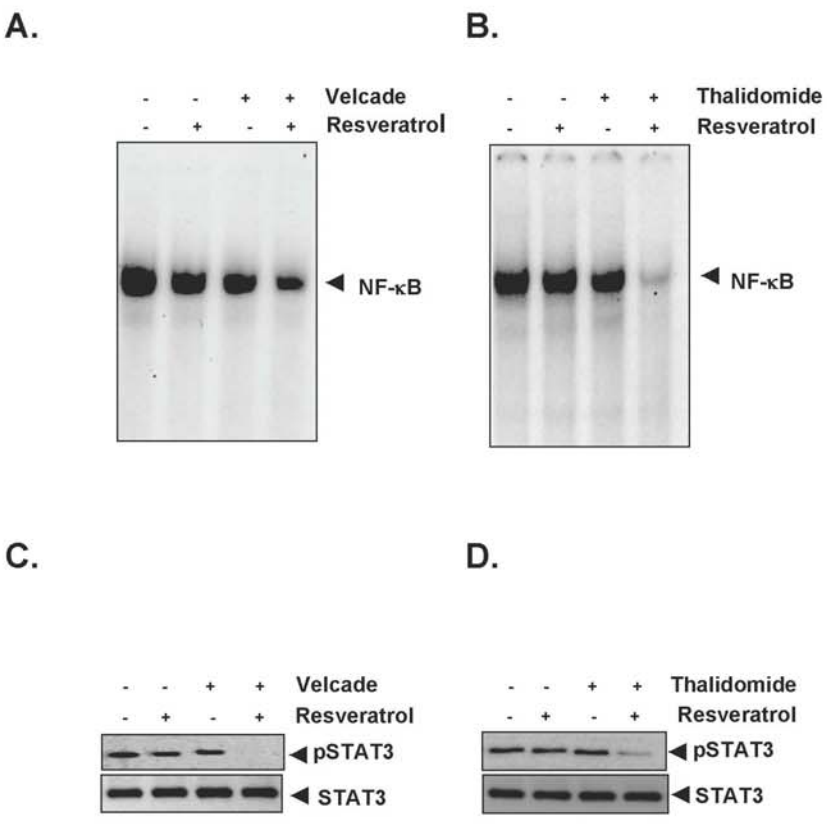

A.
U266
Time (h)
0 3 6 12 24
Resveratrol (μM)
0 10 25 50 100
NF-κB
B
MM1.S
Time (h)
0 3 6 12 24
Resveratrol (μM)
0 10 25 50 100
NF-κB
C.
RPMI 8826
Time (h)
0 3 6 12 24
Resveratrol (μM)
0 10 25 50 100
NF-κB
D.
Medium
Anti-p50
Anti-p65
Anti-P50+p65
Cold oligo
Mutant oligo
NF-κB
E.
0 3 6 12 Time (h)
GST-IκBα
IKKα
IKK β
F.
0 3 6 12 24 Time (h)
p-IκBα
β-actin
G.
0 3 6 12 24 Time (h)
p-p65
β-actin
H.
Media
Resveratrol
NF-κB (p65)
Nuclei

F4.

F5.

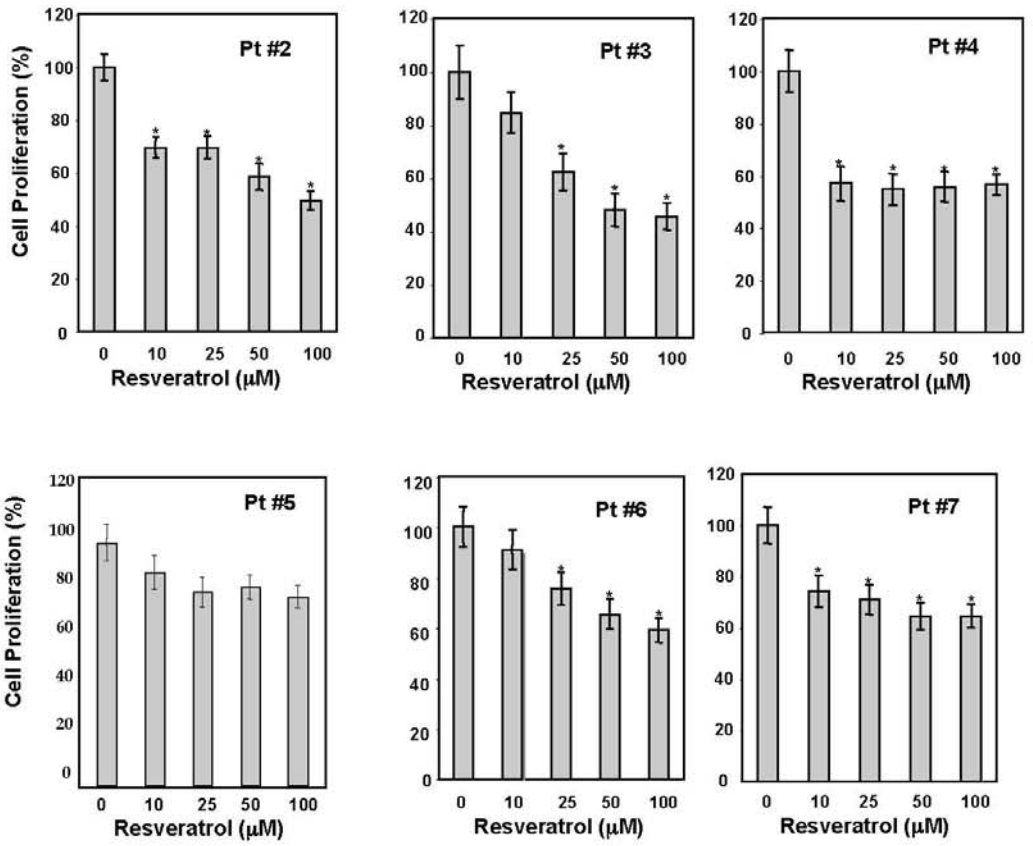

A.
0 30 60 180 Time (min)
pSTAT3
STAT3
B.
0 10 25 50 100 Resveratrol (μM)
pSTAT3
STAT3
C.
0 30 60 120 180 Time (min)
pSTAT5
STAT5
D.
Media
Resveratrol
STAT3
Nuclei
E.
IL-6
0 5 10 15 30 60 Time (min)
F.
IL-6+Resveratrol
0 0 30 60 120 180 Time(min)
pSTAT3
STAT3

F6.


A.
B.
- - + + Velcade
- + - + Resveratrol
NF-κB
- - + + Thalidomide
- + - + Resveratrol
NF-κB
C.
D.
- - + + Velcade
- + - + Resveratrol
pSTAT3
STAT3
- - + + Thalidomide
- + - + Resveratrol
pSTAT3
STAT3

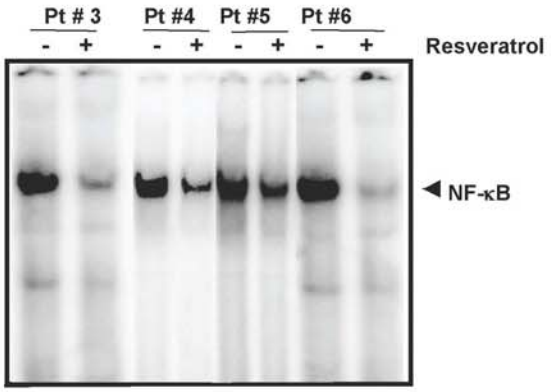
F7.

A.

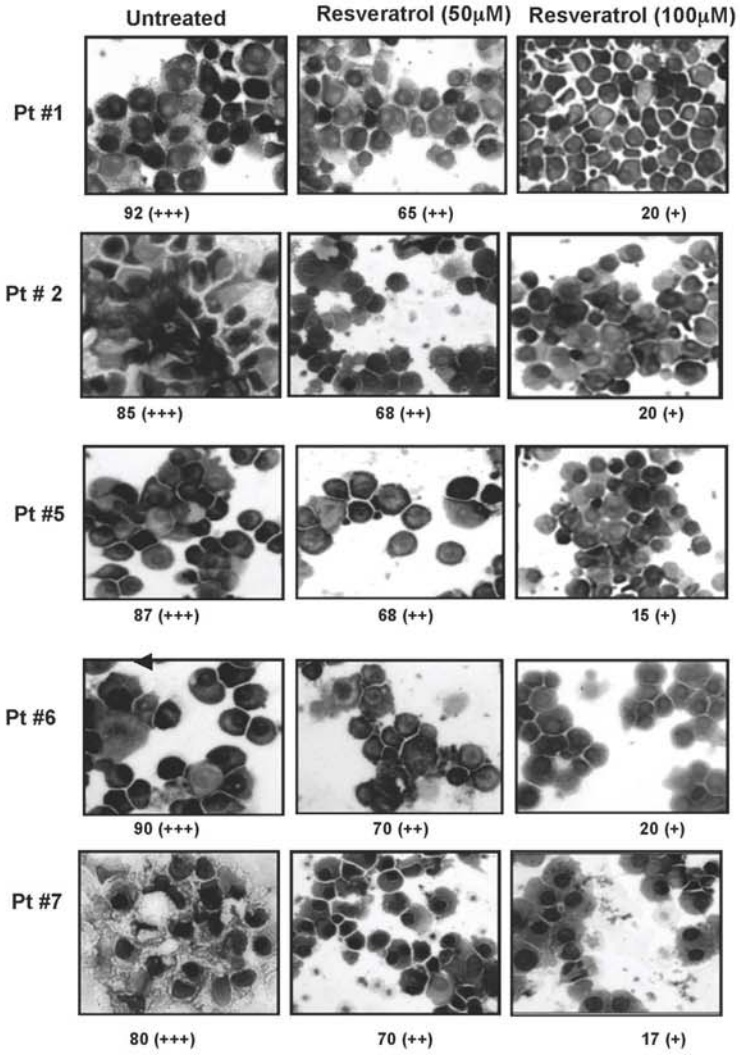

Pt #2
Pt #3
Pt #4
Pt #5
Pt #6
Pt #7
Cell Proliferation (%)
Resveratrol (μM)
0
10
25
50
100
120
100
80
60
40
20
0
*

F7.

B.

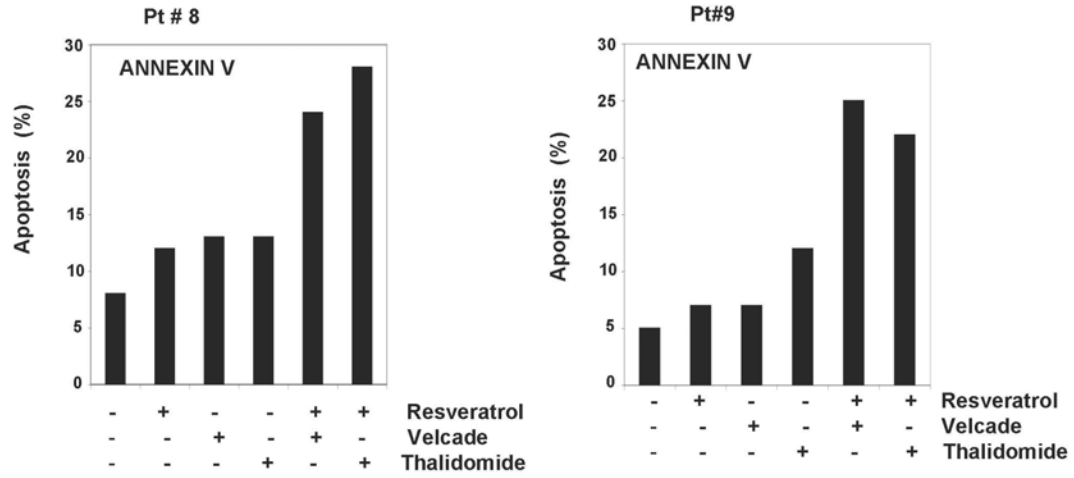

Pt # 3
Pt #4
Pt #5
Pt #6
- + - + - + - +
Resveratrol
NF-κB

C.


Untreated
Resveratrol (50μM)
Resveratrol (100μM)
Pt #1
92 (+++)
65 (++)
20 (+)
Pt # 2
85 (+++)
68 (++)
20 (+)
Pt #5
87 (+++)
68 (++)
15 (+)
Pt #6
90 (+++)
70 (++)
20 (+)
Pt #7
80 (+++)
70 (++)
17 (+)

F7.

D.


Pt # 8
ANNEXIN V
Apoptosis (%)
30
25
20
15
10
5
0
- + - - + + Resveratrol
- - + - + - Velcade
- - - + - + Thalidomide
Pt#9
ANNEXIN V
Apoptosis (%)
30
25
20
15
10
5
0
- + - - + + Resveratrol
- - + - + - Velcade
- - - + - + Thalidomide