Apoptotic Effects of Resveratrol, a Grape Polyphenol, on Imatinib-Sensitive and Resistant K562 Chronic Myeloid Leukemia Cells

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Abstract. *Aim: To examine the antiproliferative and apoptotic effects of resveratrol on imatinib-sensitive and imatinib-resistant K562 chronic myeloid leukemia cells. Materials and Methods: Antiproliferative effects of resveratrol were determined by the 3-Bis[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide inner salt (XTT) cell proliferation assay. Apoptotic effects of resveratrol on sensitive K562 and resistant K562/IMA-3 cells were determined through changes in caspase-3 activity, loss of mitochondrial membrane potential (MMP), and apoptosis by annexin V-(FITC). Results: The concentrations of resveratrol that inhibited cell growth by 50% (IC_{50}) were calculated as 85 and 122 μM for K562 and K562/IMA-3 cells, respectively. There were 1.91-, 7.42- and 14.73-fold increases in loss of MMP in K562 cells treated with 10, 50, and 100 μM resveratrol, respectively. The same concentrations of resveratrol resulted in 2.21-, 3.30- and 7.65-fold increases in loss of MMP in K562/IMA-3 cells. Caspase-3 activity increased 1.04-, 2.77- and 4.8-fold in K562 and 1.02-, 1.41- and 3.46-fold in K562/IMA-3 cells in response to the same concentrations of resveratrol, respectively. Apoptosis was induced in 58.7%- and 43.3% of K562 and K562/IMA-3 cells, respectively, in response to 100 μM resveratrol. Conclusion: Taken together these results may suggest potential use of resveratrol in CML, as well as in patients with primary and/or acquired resistance to imatinib.*

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Chronic myeloid leukemia (CML), a hematological disease, arises from the reciprocal translocation between the break-point cluster (BCR) gene on chromosome 22 and the Abelson leukemia virus oncogene (ABL) gene on chromosome 9 (1). This translocation generates the Philadelphia (Ph) chromosome encoding BCR-ABL fusion protein which has constitutively activated tyrosine kinase activity. This protein has an ATP-binding pocket and a substrate-binding site (2). When ATP binds to the ATP-binding pocket of this protein, ATP constitutively phosphorylates its substrate, therefore, BCR-ABL becomes active. BCR-ABL induces many signaling pathways contributing to cell survival and causing the disruption of genetic stability (3). In the clinic, tyrosine kinase inhibitors are generally used for CML therapy. Imatinib mesylate, a first-generation tyrosine kinase inhibitor, binds to the ATP-binding pocket of BCR-ABL, preventing the phosphorylation of the substrate, and then also activation of BCR-ABL. Nowadays, imatinib is used for first-line therapy in CML due to its great efficacy (4). However, the therapeutic efficacy of imatinib is reduced by the emergence of resistance towards this drug (5). Point mutations in the kinase domain of BCR-ABL, activation of membrane transporters, overexpression of BCR-ABL, aberrant ceramide metabolism, and the presence of CML stem cells in a quiescent state are the main molecular mechanisms underlying drug resistance in CML (6, 7). To overcome this resistance, more effective tyrosine kinase inhibitors are being developed (8), but drug resistance and adverse effects of new agents are still important problems. Therefore, natural products have been investigated due to their potential in cancer prevention and treatment (9).

Resveratrol (3,5,4'-*trans*-trihydroxystilbene), one of the most important natural products, is produced by numerous plants, especially by red grapes, in order to protect themselves from detrimental environmental conditions (10, 11). Resveratrol has been reported to have the potential to inhibit cancer initiation, promotion, and progression (12). It has also anti-metastatic, anti-inflammatory, chemopreventive, radiosensitizer, and even

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chemosensitizer effects (13-15). Through binding directly to DNA or RNA, resveratrol protects DNA from mutagens (12). Furthermore, resveratrol can induce apoptosis *via* inhibiting DNA topoisomerase II, nuclear factor kappa B (NF-κB) or signal transducer and activator of transcription 3 (STAT3) pathways, and by repressing the expressions of anti-apoptotic genes such as B-cell lymphoma-extra large (BCL-XL), B-cell lymphoma 2 (BCL-2), and X-linked inhibitor of apoptosis protein (XIAP) (12, 16).

The aim of this study was to examine the antiproliferative effects of resveratrol on imatinib-sensitive and imatinib-resistant, Philadelphia-positive K562 CML cells we developed in our laboratory over three years.

Materials and Methods

Chemicals. Resveratrol was obtained from Sigma Aldrich (St. Louis, MO, USA). The stock solution of resveratrol was dissolved in dimethylsulfoxide (DMSO) at a concentration of 10 M, stored at –20˚C, and diluted in cell culture medium. Penicillin-streptomycin, RPMI 1640, and fetal bovine serum were obtained from Invitrogen (Paisley, UK).

Cell lines and culture conditions. Human K562 CML cells were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). K562 cells were maintained in RPMI 1640 growth medium containing 10% fetal bovine serum and 1% penicillin-streptomycin at 37˚C in 5% CO_2. K562 cells grown in RPMI 1640 were exposed to increasing concentrations of imatinib, starting with 50 nM. At the end of this process, we obtained cells resistant to 3 μM imatinib and named them K562/IMA-3 cells.

Measurement of cell growth by 3-bis[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide inner salt (XTT) assay. The resveratrol concentration inhibiting cell growth by 50% (IC_{50}) was determined by XTT cell proliferation assay. In brief, 2×10^4 cells/200 μl/well were seeded into 96-well plates containing 100 μl of the growth medium in the absence or presence of increasing concentrations of resveratrol, and were then incubated at 37˚C with 5% CO_2. After a 72 h incubation period, cells were treated with 50 μl XTT for 4 h. Then, plates were read at 492 nm wavelength by an ELISA reader (Thermo Electron Corporation Multiskan Spectrum, Vantaa, Finland). Finally, the IC_{50} value of resveratrol was calculated according to the cell proliferation plots.

Evaluation of apoptosis.
Determination of the changes in caspase-3 activity: Changes in caspase-3 activity of the cells, as an important sign of apoptosis, were determined by caspase-3 colorimetric assay kit as described elsewhere (BioVision, Edmonton, AB, Canada) (17). This assay is based on spectrophotometric detection of the chromophore p-nitroanilide (pNA) after cleavage from the labelled substrate acetyl-Asp-Glu-Val-Asp (DEVD)-pNA which is a specific substrate for caspase-3 enzyme. Briefly, the cells (1×10^6 cells/2 ml/well) induced to undergo apoptosis by treatment with resveratrol, were collected by centrifugation at 800 ×*g* for 10 min. The cells were lysed by adding 50 μl of chilled cell lysis buffer and incubated on ice for 10 min before centrifugation at 10,000 ×*g* for 1 min. Since the proteins were in supernatant, the upper part of the mixture was transferred to new Eppendorf tubes and the reaction mixture was prepared in 96-well plates by adding 50 μl of 2× reaction buffer [containing 10 mM dithiothreitol (DTT)], 50 μl of sample, and 5 μl of DEVD-pNA substrate, and the plates were incubated for 2 h at 37˚C with %5 CO_2. At the end of this period of time, the plate was read at 405 nm wavelength by an ELISA reader (Thermo Electron Corporation Multiskan Spectrum). The absorbance values were normalized to protein concentrations determined by the Bradford assay as decribed (7).

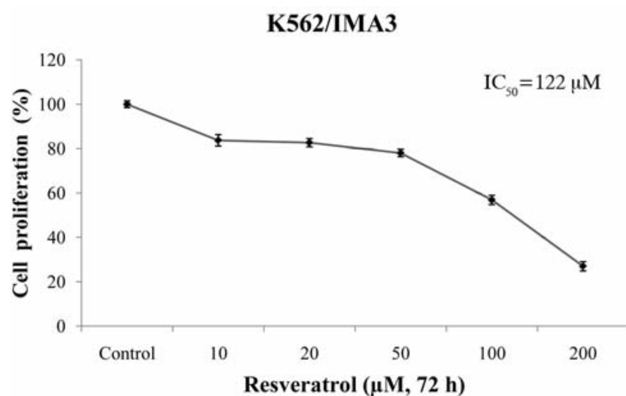

Figure 1. *Cytotoxic effects of resveratrol on K562/IMA-3 cells. Cytotoxicity was determined by the Bis[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide inner salt (XTT) cell proliferation assay in a 72 h culture. The XTT assays were performed using triplicate samples in at least three independent experiments. Values are the arithmetic means of triplicates. The error bars represent the standard deviation.*

Detection of loss of mitochondrial membrane potential (MMP): The loss of MMP is an indicator of apoptosis and was detected by JC-1 mitochondrial membrane potential detection kit as described elsewhere (Cayman Chemicals, Ann Arbor, MI, USA) (18). Imatinib-resistant and sensitive K562 cells (5×10^5) were seeded into 96-well plates in 100 μl growth medium. These cells were treated with different concentrations of resveratrol and incubated for 72 h at 37˚C with 5% CO_2. After 72 h, cells were treated with 10 μl JC-1 staining solution and incubated for 20 min in 5% CO_2. The cells were then collected and centrifuged for 5 min at 400 ×*g*. The supernatant was removed and cell pellet was washed with 1×phosphate buffered saline (PBS) before centrifugation at 400 ×*g* for 5 min. This step was repeated twice. Finally, 100 μl assay buffer was added to the cells. The aggregate red forms accumulated in the inner mitochondria have absorption/emission maxima of 585/590 nm and the monomeric green forms released into the cytoplasm due the loss of the MMP have absorption/emission maxima of 510/527 nm. The plate was read at these wavelengths by fluorescence ELISA reader (Thermo Varioskan Spectrum). Green/red (510/585) absorption values were calculated in order to determine the changes in MMP.

Detection of early apoptosis by Annexin V-Fluorescein isothiocyanate (FITC): K562/IMA-3 and sensitive K562 (1×10^6) cells were seeded onto 6-well plates and treated with different

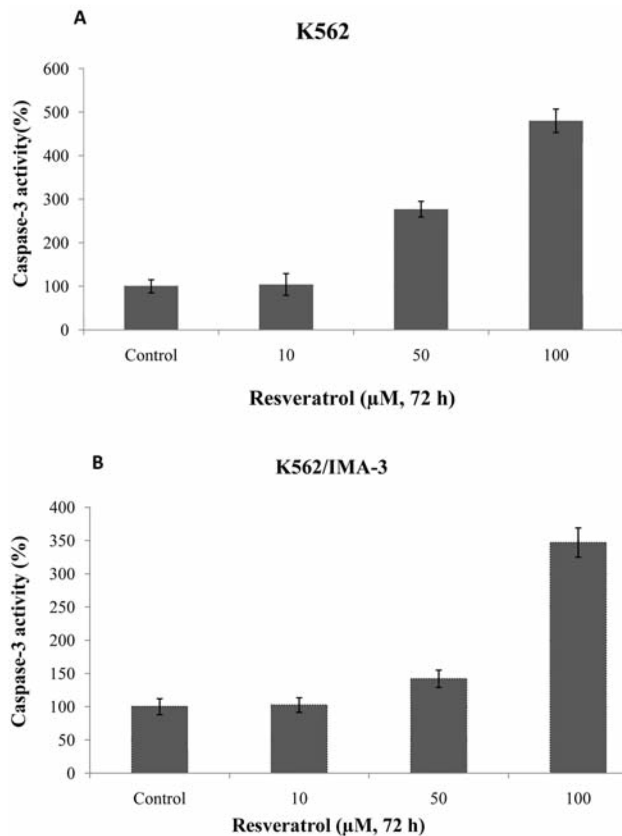

Figure 2. *Changes in caspase-3 activity in response to resveratrol in K562 (A) and K562/IMA-3 (B) cells. Values are the arithmetic means of three independent experiments. The error bars represent the standard deviations.*

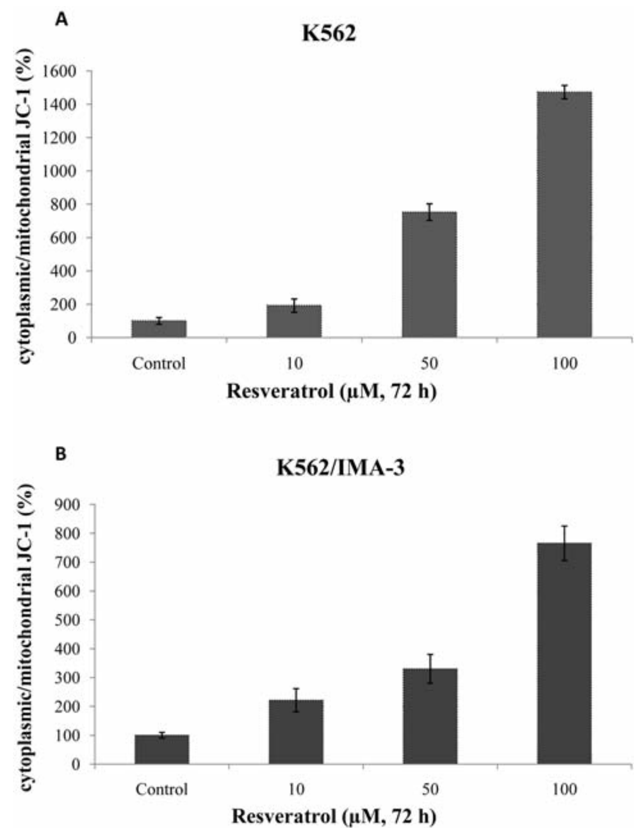

Figure 3. *Changes in cytoplasmic/mitochondrial JC-1 in K562 (A) and K562/IMA-3 (B) cells in response to resveratrol. Values are the arithmetic means of three independent experiments. The error bars represent the standard deviations.*

concentrations of resveratrol. Cells were incubated for 72 h with 5% CO_2. Then the cells were centrifuged at 800 ×*g* for 5 min. Supernatants were removed and binding buffer was added to the pellets. Cells were treated with 5 μl of FITC and 5 μl of propidium iodide (PI). The mixture was incubated for 15 min in the dark. The samples were then analyzed for AnnexinV-FITC binding by flow cytometry (Ex=488 nm; Em=530 nm) using FITC signal detector and PI staining emission signal detector. H_2O_2, FITC and PI were also added to cells and were used as positive control.

Results

Cellular growth assessment via XTT cell proliferation assay. Our previous findings demonstrated that K562/IMA-3 cells were around 100-fold more resistant to imatinib as compared to sensitive K562 cells (17). Cytotoxic effects of resveratrol on K562/IMA-3 and sensitive K562 cells were determined by XTT cell proliferation assay. We previously determined the IC_{50} value of resveratrol for K562 cells as being 85 μM (18), while that for K562/IMA-3 was calculated from cell proliferation plots and found to be 122 μM (Figure 1). These results demonstrated that K562/IMA-3 cells were only 1.4-fold more resistant to resveratrol than were K562 cells. Resveratrol reduced cell proliferation of both cell types in a dose dependent manner.

Resveratrol increases caspase-3 activity in a dose dependent manner in both K562 and K562/IMA-3 cells. In order to examine the changes in caspase-3 activity in response to resveratrol, K562 and K562/IMA-3 cells were treated with different concentrations of resveratrol for 72 h. The results demonstrated that caspase-3 activity in K562 cells treated with 10, 50 and 100 μM resveratrol was 1.09-, 2.77- and 4.88-fold, respectively, as compared to the untreated control group (Figure 2A). On the other hand, the same concentrations of resveratrol increased caspase-3 activity in K562/IMA3 cells to 1.02-, 1.41- and 3.46-fold that of the control, respectively (Figure 2B). Resveratrol increased caspase-3 activity in a dose dependent manner with no significant changes in K562 and K562/IMA-3 cells.

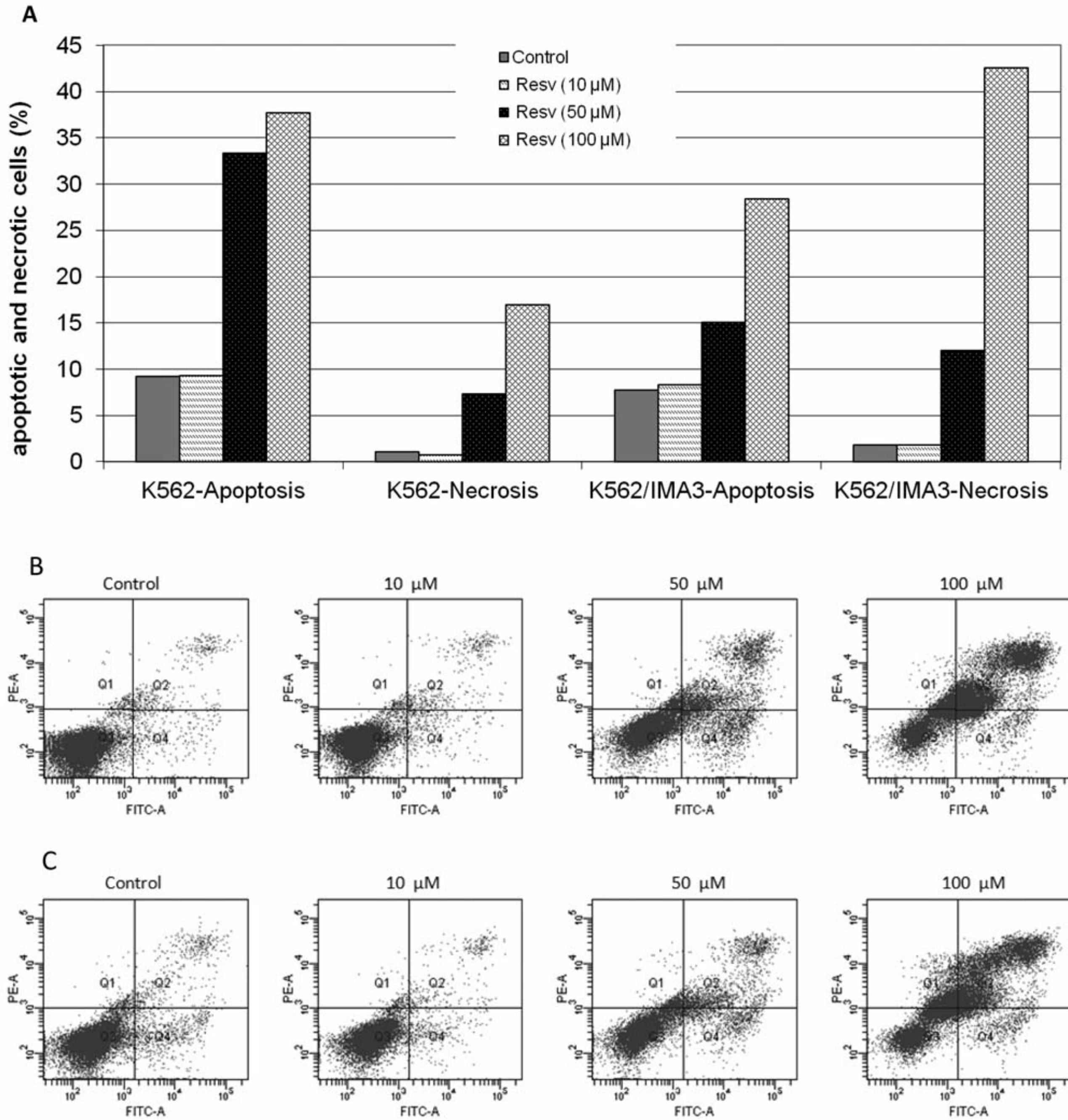

Figure 4. *Changes in populations of apoptotic and necrotic K562 (A, B) and K562/IMA-3 cells (A, C) in response to resveratrol.*

Resveratrol induces loss of MMP in both K562 and K562/IMA-3 cells in a dose-dependent manner. In order to confirm the findings of caspase-3 activity, the same concentrations of resveratrol were applied to K562 and K562/IMA-3 cells and the loss of MMP was determined. The results revealed that loss of MMP was 1.91-, 7.42-, and 14.73-fold in K562 cells treated with 10, 50, and 100 μM resveratrol, respectively, as compared to untreated controls (Figure 3A). The same concentrations of resveratrol led to a loss of MMP of 2.21-, 3.30-, and 7.65-fold in K562/IMA-3 cells, respectively, as compared to untreated controls (Figure 3B).

Resveratrol induces apoptosis of both K562 and K562/IMA-

3 cells in a dose-dependent manner. In order to confirm changes in caspase-3 activity and loss of MMP in response to resveratrol, we examined the changes in the apoptotic cell population in K562 and K562/IMA-3 cells exposed to increasing concentrations of resveratrol by Annexin-V apoptosis assay and flow cytometry. The results demonstrated that 9.3% and 8.3% of the K562 and K562/IMA3 cell populations were in apoptosis, respectively, in response to 10 μM resveratrol (Figure 4). On the other hand, 100 μM resveratrol induced apoptosis in 37% and 28% of K562 and K562/IMA-3 cells, respectively (Figure 4). Resveratrol increased apoptotic cell population in a dose dependent manner in both cell lines but there was no significant change in the apoptotic populations of K562 and K562/IMA-3 cells.

Discussion

Imatinib mesylate is a first-generation tyrosine kinase inhibitor suppressing the activity of BCR-ABL fusion protein, the initiator of CML. It is accepted as the gold standard for treatment of CML patients. Imatinib not only targets the BCR-ABL fusion protein but also the tyrosine-protein kinase kit (c-KIT) and the platelet-derived growth factor receptor (PDGFR) (19, 20). Imatinib is a competitive inhibitor which specifically recognizes and binds to the ATP binding site of the protein, and prevents phosphorylation of downstream substrates of the oncogenic protein (21). The most important problem in the treatment of CML by imatinib, as observed for other different types of cancer, is the development of resistance or intolerance to imatinib (6, 22). Unfortunately, resistance is still an important problem for the treatment of CML with novel agents, nilotinib and dasatinib, which are more effective than imatinib (23). Recent studies focused on the reversal of the resistance and the investigation of natural products that may have potential to be used as a supplementary to anticancer agents. In this study, we aimed at examining the anticancer potential of resveratrol, a plant-derived phytochemical, on both imatinib-sensitive and -resistant K562 cells.

Different groups showed that resveratrol induces apoptosis, and has anti-metastatic, anti-inflammatory, cardioprotective, chemopreventive and radiosensitizer effects (13-15). More importantly, resveratrol inhibits initiation and progression of different types of cancer (12). Resveratrol triggers apoptosis in cancer cells through both intrinsic and extrinsic pathways. Gledhill *et al*. showed that resveratrol inhibits F0F1 ATP synthase, which is involved in cellular ATP synthesis, and induces apoptosis *via* mitochondrial dysfunction (24). While resveratrol reduces expression of breast cancer resistance protein (BCRP) in breast cancer (25), treatment of prostate cancer cells with resveratrol increases the expression of Bcl-2 associated X (BAX), Bcl-2 associated death promoter (BAD), BH3 domain only death agonist (BID), and Bcl-2 homologous antagonist/killer (BAK) apoptotic genes and of *p53*, the guardian of the genome (26). Our group recently showed that resveratrol triggers apoptosis through up-regulating longevity assurance (LASS) genes that generate apoptotic ceramides and by down-regulating sphingosine kinase-1 and glucosylceramide synthase genes that convert apoptotic ceramides to antiapoptotic sphingosine-1-phosphate and glucosylceramide lipids, respectively, in acute myeloid leukemia (18) and CML cells (27). Furthermore, Ragione *et al*. demonstrated that resveratrol inhibits carcinogenesis and induces differentiation of CML and acute myeloid leukemia cells (28, 29).

Our results demonstrated that resveratrol has antiproliferative and apoptotic effects on K562 CML cells. More importantly, we have also shown for the first time that resveratrol has significant apoptotic and antiproliferative effects on K562/IMA-3 cells that are ~100-fold more resistant to imatinib as compared to their parental sensitive counterparts (17).

Taken together, the results of this study indicate that resveratrol may have therapeutic potential and suggest that consumption of resveratrol-containing products may help CML treatment of both imatinib-sensitive and -resistant cases.

Conflict of Interest

We confirm that we do not have any conflict of interest, nor do we have any financial or personal relationships with other people or organisations that could inappropriately influence (bias) our work.

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