# Resveratrol-caused apoptosis of human prostate carcinoma LNCaP cells is mediated via modulation of phosphatidylinositol 3′-kinase/Akt pathway and Bcl-2 family proteins

**Moammir H. Aziz,[1] Minakshi Nihal,[1] Vivian X. Fu,[2] David F. Jarrard,[2] and Nihal Ahmad[1]**

**Departments of [1]Dermatology and [2]Surgery, University of Wisconsin, Madison, Wisconsin**

## Abstract

**Prostate cancer is a major health problem in the U.S. and the available treatment and surgical options have proven to be inadequate in controlling the mortality and morbidity associated with this disease. It is therefore necessary to intensify our efforts to better understand this disease and develop novel approaches for its prevention and treatment. This study was conducted to evaluate the chemopreventive/antiproliferative potential of resveratrol (*trans*-3,4′,5,-trihydroxystilbene) against prostate cancer and its mechanism of action. Treatment with resveratrol (0–50 μmol/L for 24 hours) resulted in a significant (*a*) decrease in cell viability, (*b*) decrease of clonogenic cell survival, (*c*) inhibition of androgen (R1881)-stimulated growth, and (*d*) induction of apoptosis in androgen-responsive human prostate carcinoma (LNCaP) cells. Interestingly, at similar concentrations, resveratrol treatment did not affect the viability or rate of apoptosis in normal human prostate epithelial cells. Furthermore, our data showed that resveratrol-treatment resulted in significant dose-dependent inhibition in the constitutive expression of phosphatidylinositol 3′-kinase and phosphorylated (active) Akt in LNCaP cells. Resveratrol treatment for LNCaP cells was also found to result in a significant (*a*) loss of mitochondrial membrane potential, (*b*) inhibition in the protein level of antiapoptotic Bcl-2, and (*c*) increase in proapoptotic members of the Bcl-2 family, i.e., Bax, Bak, Bid, and Bad. Taken together, our data suggested that resveratrol causes an inhibition of phosphatidylinositol 3′-kinase/Akt activation that, in turn, results in modulations in Bcl-2 family proteins in such a way that the apoptosis of LNCaP cells is promoted. Based on these studies, we suggest that resveratrol could be developed as an agent for the management of prostate cancer. [Mol Cancer Ther 2006;5(5):1335–41]**

## Introduction

Prostate cancer is the most common cancer of men in the U.S. and, next only to lung cancer, is the second leading cause of cancer-related deaths in American males (1). According to an estimate, in the U.S. alone, 234,600 new cases of prostate cancer will be diagnosed in the year 2006, and 27,350 prostate cancer–related deaths are predicted (1). The existing treatment approaches and surgical intervention have not been able to effectively manage this dreaded cancer, and metastatic disease frequently redevelops even after surgery. Therefore, continuing efforts are ongoing to explore novel mechanism-based targets and strategies for the management of prostate cancer.

Chemoprevention, via ingestion of natural or synthetic agents with low toxicity that are able to suppress, delay, or reverse carcinogenesis, is being considered as a new dimension in the management of neoplasia including prostate cancer (2, 3). In the recent past, *trans*-resveratrol (*trans*-3,4′,5-trihydroxystilbene) has been shown to inhibit cancer initiation, promotion, and progression (2, 4–9). Resveratrol is a phytoestrogen present in the skin of red grapes and various other food products, with structural similarity to estradiol and diethylstilbestrol (2, 4–9). Studies have shown that each gram of fresh red grape skin contains 50 to 100 μg of resveratrol, and its concentration in red wine ranges from 10 to 20 μmol/L (10). As a consequence, resveratrol is being considered as an excellent candidate agent for cancer chemoprevention (2, 4–9). Studies have shown that resveratrol imparts chemopreventive/antiproliferative effects against several cancer types including prostate cancer, both *in vitro* as well as *in vivo* (2, 4–9, 11–15). However, the mechanisms associated with the antiproliferative/chemopreventive effects of resveratrol are not well established.

The phosphatidylinositol 3′-kinase (PI3K)/Akt pathway is known to play an important role in cell survival (inhibition of apoptosis) and PI3K activity has been linked to a variety of human cancers (16–20). Protein kinase B, also known as Akt, is a downstream kinase of PI3K and is known to phosphorylate and regulate the function of many cellular proteins involved in processes that include metabolism, apoptosis, and proliferation (16–20). Constitutive Akt signaling is believed to promote proliferation and increased cell survival, thereby contributing to cancer

Received 12/15/05; revised 3/7/06; accepted 3/24/06.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

**Requests for reprints:** Nihal Ahmad, Department of Dermatology, University of Wisconsin, 1300 University Avenue, Medical Science Center B-25, Madison, WI 53706. Phone: 608-263-5359; Fax: 608-263-5223. E-mail: nahmad@wisc.edu

Copyright © 2006 American Association for Cancer Research.

doi:10.1158/1535-7163.MCT-05-0526



Downloaded from mct.aacrjournals.org on July 27, 2017. © 2006 American Association for Cancer Research.

progression; recent studies have shown that Akt is constitutively active in several types of human cancers (16–20). The PI3K/Akt pathway has been shown to play a critical role in prostate cell proliferation and survival (21). Several lines of evidence have suggested that PI3K/Akt and PTEN could modulate androgen-induced cell growth and androgen receptor–mediated transcription in prostate cancer cells, suggesting a potential link between the PI3K/Akt and androgen pathways (21–24). Studies have also suggested that the PI3K/Akt pathway plays a role in the regulation of Bcl-2 family proteins, which are believed to be important targets for cancer drug development (25–27). All these studies suggest that prostate cancer cells may be uniquely sensitive to the effects of PI3K pathway inhibitors. Thus, there is guarded optimism that novel approaches directed against the PI3K pathway could be useful for the management of prostate cancer (18–23).

In this study, we have shown that resveratrol (*a*) inhibits the PI3K/Akt pathway, (*b*) down-regulates antiapoptotic protein Bcl-2, and (*c*) up-regulates proapoptotic members of Bcl-2 family of proteins during its antiproliferative effects in androgen-responsive human prostate carcinoma LNCaP cells.

## Materials and Methods

### Reagents

Resveratrol (>99% pure) was purchased from Alexis Biochemicals (San Diego, CA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma Chemical Co. (St. Louis, MO). The antibodies were obtained from the following vendors: Cell Signaling Technology, Beverly, MA (PI3K/p85, phospho-Ser$^{473}$-Akt, and Akt); Santa Cruz Biotechnology, Santa Cruz, CA (PI3K/p110 and β-actin); Biosource International, Camarillo, CA (Bcl-2, Bax, Bad, and Bak); Upstate Biotechnology, Lake Placid, NY (anti-mouse and anti-rabbit secondary conjugated horseradish peroxidase antibody). APO-Direct apoptosis kit was obtained from Phoenix Flow Systems (San Diego, CA). Bicinchoninic acid protein assay kit was obtained from Pierce Biotechnology (Rockford, IL). PAGEr precast Tris-Glycine gels was purchased from Cambrex Bio Science (Rockland, ME). All other chemicals were purchased in the purest form commercially available.

### Cell Culture

The androgen-responsive human prostate carcinoma LNCaP cells were obtained from American Type Culture Collection (Rockville, MD). The cells were cultured in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) with 10% fetal bovine serum, and 1% penicillin-streptomycin. Human prostate epithelial cells (HPEC) were established on collagen-coated dishes in Ham's F-12 supplemented medium containing 1% fetal bovine serum as described earlier (28, 29). Human prostate epithelial tissue samples were obtained from cystoprostatectomy specimens (of patients ages 45–60) that did not contain prostate cancer. The protocol of the procurement of tissue was approved by the Institutional Review Board for human subject research.

### Cell Growth/Cell Viability

The effect of resveratrol on the viability of HPEC and LNCaP cells was determined by trypan blue dye exclusion and MTT assays. Briefly, the cells were plated at a density of $1 \times 10^5$ in six-well plates and in 2 mL complete medium containing different concentrations of resveratrol (1–50 μmol/L in DMSO) or vehicle alone. After incubation for 24 hours, cells were collected and an aliquot of cell suspension was mixed with an equal volume of trypan blue and cells were counted under the microscope.

For the MTT assay, cells were plated at a density of $2 \times 10^3$ cells per well in 200 μL of complete medium containing different concentrations of resveratrol (1–50 μmol/L in DMSO) or vehicle alone, in a 96-well microtiter plate. Each treatment was repeated in 10 wells. The cells were incubated for 24 hours at 37°C in a humidified chamber at the end of which MTT reagent (4 μL, 5 mg/mL in PBS) was added to each well and incubated for 2 hours. The microtiter plate containing the cells was centrifuged at 1,800 rpm for 5 minutes at 4°C. The MTT solution was removed from the wells by aspiration and the formazan crystals were dissolved in DMSO (150 μL). Absorbance was recorded at 540 nm wavelength.

### Soft Agar Colony Formation Assay

The effect of resveratrol on colony formation ability of the LNCaP cells was assessed by soft agar colony formation assay. The assay was done in six-well plates; in each well, 2 mL of 0.5% agar (in culture medium) was layered in the bottom followed by 1 mL of 0.38% agar as the top layer. Approximately 2,000 cells were then plated over the top layer. The cells were treated with resveratrol (0–50 μmol/L) and maintained at 37°C in a humidified 5% $CO_2$ atmosphere. After 14 days, the number of colonies were counted under an inverted phase contrast Olympus $1 \times 70$ microscope at 4× magnification and photographed.

### Effect on Androgen-Stimulated Cell Growth

The effect of resveratrol on R1881 (synthetic androgen)-stimulated growth of LNCaP cells was determined by MTT assay as described elsewhere (30). Briefly, cells were plated at a density of $2 \times 10^3$ cells per well in 96-well plates, and the following day, the medium was removed and cells were washed with PBS. Fresh medium, similar to the normal growth medium, except that it contained 10% charcoal stripped serum and was phenol red–free, was added. The cells were starved of hormone for 48 hours after which cells were stimulated with R1881 (0.1 nmol/L). The cells were treated with R1881 and resveratrol (1–50 μmol/L in DMSO) for 24 hours and MTT assay was done as described above.

### Quantification of Apoptosis by Flow Cytometry

The extent of apoptosis assay was done with the APO-Direct assay kit (Phoenix Flow Systems) as per the manufacturer's protocol. Briefly, the cells were grown at a density of $1 \times 10^6$ cells in six-well culture dishes and were treated with different concentrations of resveratrol (0–50 μmol/L in DMSO) for 24 hours and maintained at 37°C in a humidified 5% $CO_2$ atmosphere. Following treatments, the cells were gently trypsinized and added to the culture medium and pelleted by centrifugation. The cell pellet was



Downloaded from mct.aacrjournals.org on July 27, 2017. © 2006 American Association for Cancer Research.

resuspended in PBS, and washed, and the cells were fixed in ethanol (90% v/v). The cells were then washed with PBS, and labeled with terminal nucleotidyl transferase enzyme and FITC-dUTP for 60 minutes at 37°C, followed by washing with PBS and resuspending in propidium iodide/RNase A solution. Labeled cells were analyzed by flow cytometry and the analyses were done using Cell Quest software (BD Biosciences, San Jose, CA).

### JC-1 Mitochondrial Membrane Potential Detection Assay

We used APO-LOGIC JC1 kit (Cell Technology, Inc., Minneapolis, MN) for *in situ* detection of mitochondrial membrane transition events in live cells, which provide an early indication of the initiation of cellular apoptosis. The collapse in the electrochemical gradient across the mitochondrial membrane ($\psi$) was measured using a fluorescent cationic dye 5,5′6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzamidazolocarbocyanin iodide, known as JC-1. For this assay, the growing cells (60% confluence) were treated with resveratrol (1–50 μmol/L) or vehicle (DMSO) for 24 hours. Cells were collected and suspended in 0.5 mL 1× APO LOGIC JC-1 reagent followed by incubation at 37°C in a 5% $CO_2$ incubator for 15 minutes. The cells were then collected by centrifugation and the pellet was washed with 2 mL assay buffer (1×) and then resuspended in 0.5 mL assay buffer. The cells were analyzed by fluorescence microscopy using a dual bandpass filter designed to simultaneously detect fluorescein and rhodamine or fluorescein and Texas red. In live nonapoptotic cells, the mitochondria appear red following aggregation of APO LOGIC JC-1 reagent. In apoptotic and dead cells, the dye remains in its monomeric form and appears green.

### Preparation of Cell Lysates and Western Blot Analysis

The cells were harvested at 24 hours following resveratrol treatment as described above and washed with cold PBS (10 mmol/L; pH 7.4). The cells were incubated in ice-cold lysis buffer [50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L EGTA, 1 mmol/L EDTA, 20 mmol/L NaF, 100 mmol/L $Na_3VO_4$, 0.5% NP40, 1% Triton X-100, and 1 mmol/L phenylmethylsulfonyl fluoride (pH 7.4)] with freshly added protease inhibitor cocktail (Protease Inhibitor Cocktail Set III, Calbiochem, La Jolla, CA) over ice for 30 minutes. The cells were scraped and the lysate was collected in a microfuge tube and passed through a 21.5-gauge needle to break up the cell aggregates. The lysate was cleared by centrifugation at 14,000 × *g* for 15 minutes at 4°C, and the supernatant (total cell lysate) was collected, aliquoted, and stored at −70°C. The protein concentration was determined using the bicinchoninic acid protein assay (Pierce Biotechnology) as per the manufacturer's protocol.

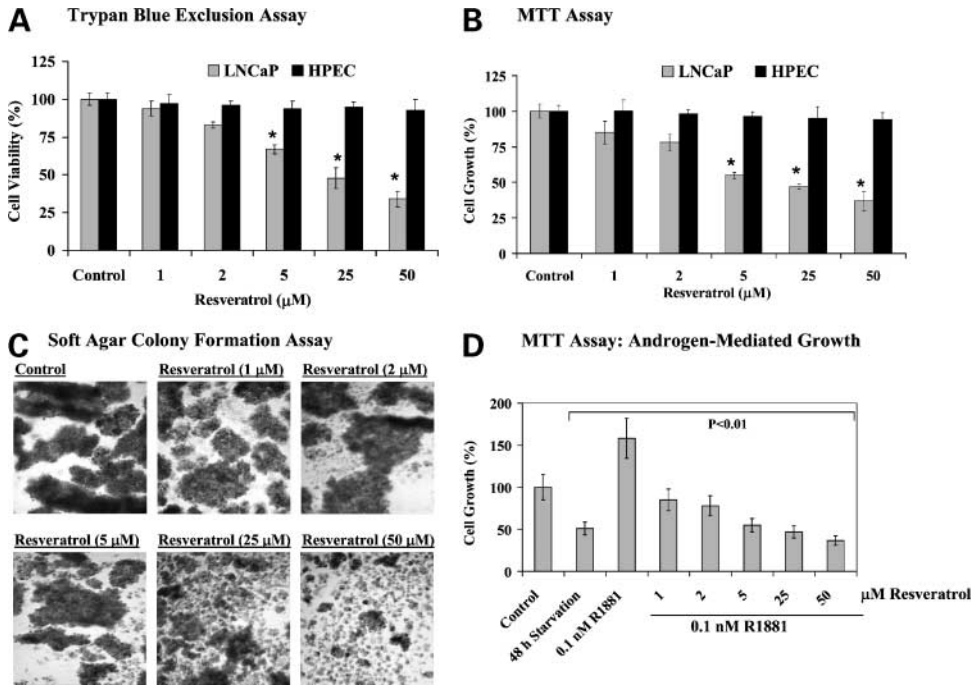


**Figure 1.** Effect of resveratrol on the viability and clonogenic survival of LNCaP and/or HPEC cells. **A,** trypan blue exclusion assay. The cells were treated with resveratrol (0–50 μmol/L) for 24 h and the viability of cells was determined by trypan blue exclusion assay. *Columns,* mean percentage of cell viability from three experiments conducted in triplicate; *bars,* ±SE (*, $P < 0.01$). **B,** MTT assay. The cells were treated with resveratrol (0–50 μmol/L) and cell growth was determined by MTT assay. *Columns,* mean percentage of reduction in cellular metabolic activity from three experiments in which each treatment was done in at least 10 wells, *bars,* ±SE (*, $P < 0.01$); **C,** soft agar colony formation assay. The growing cells were plated on a thin layer of agar in culture medium and treated with resveratrol (0–50 μmol/L). At 14 d posttreatment, plates were assessed for size and number of colonies (original magnification, ×4). Colonies (in a representative field) are indicated. Results from a representative experiment repeated thrice with similar results. **D,** MTT assay for androgen-stimulated cell growth. The cells were starved for 48 h followed by the addition of 0.1 nmol/L R1881 in the absence and presence of resveratrol (0–50 μmol/L), and cell growth was determined by MTT assay. *Columns,* mean percentage of reduction in cellular metabolic activity from three experiments in which each treatment was done in at least 10 wells; *bars,* ±SE (*, $P < 0.01$).



Downloaded from mct.aacrjournals.org on July 27, 2017. © 2006 American Association for Cancer Research.

For immunoblot analysis, appropriate amounts of protein (20–30 μg) were resolved over 10% to 15% tris-glycine polyacrylamide gel and transferred onto nitrocellulose membranes. The nonspecific sites were blocked by incubating the blot with 5% nonfat dry milk in buffer (10 mmol/L Tris, 100 mmol/L NaCl, and 0.1% Tween 20) for 1 hour at room temperature or overnight at 4°C. The blot was washed with wash buffer (10 mmol/L Tris, 100 mmol/L NaCl, and 0.1% Tween 20) for 2 × 10 minutes and incubated overnight with appropriate primary antibody at dilutions specified by the manufacturer. The blot was washed for 2 × 10 minutes followed by incubation with the corresponding secondary antibody horseradish peroxidase conjugate (Amersham Life Science, Inc., Arlington Heights, IL) at 1:2,000 dilution for 1 hour at room temperature. The blot was washed twice in wash buffer for 10 minutes each and four times for 5 minutes each. The proteins were detected by chemiluminescence and autoradiography with XAR-5 film (Amersham Life Science). For every immunoblot, equal loading of protein was confirmed by stripping the blot and reprobing with β-actin antibody. The quantification of protein was done by digital analyses of protein bands (TIFF images) using UN-SCAN-IT software (Silk Scientific; Orem, UT).

## Results

### Resveratrol Treatment Resulted in a Significant Inhibition in the Viability, Growth, and Colonogenic Survival of LNCaP Cells

The effects of resveratrol on the viability and growth of LNCaP cells were studied using trypan blue exclusion assay and MTT assays, respectively. As shown in Fig. 1A and B, our data clearly showed that resveratrol treatment resulted in a dose-dependent decrease in the viability as well as growth of LNCaP cells, respectively. However, the interesting observation of this study was that resveratrol treatment did not affect the viability/growth of normal HEPC cells, even at higher resveratrol concentrations (50 μmol/L).

Because the colony formation ability of cells is considered to be a reasonably good predictor of their *in vivo* proliferative potential, we examined the effect of resveratrol on the clonogenic survival of LNCaP cells. Our data showed that resveratrol treatment resulted in a significant inhibition in the colony formation ability of LNCaP cells (Fig. 1C).

### Resveratrol Treatment Resulted in a Significant Inhibition in Androgen (R1881)-Stimulated Growth of LNCaP Cells

Because LNCaP cells are androgen sensitive, we determined the effect of resveratrol on androgen-stimulated growth. For this purpose, the LNCaP cells were starved of androgen for 48 hours by using charcoal-stripped serum (cFBS), and subsequently stimulated with 0.1 nmol/L of synthetic androgen R1881 for 48 hours in the presence and absence of resveratrol. As shown in Fig. 1D, our data showed that R1881-stimulated growth is significantly inhibited by resveratrol.

### Resveratrol Treatment Resulted in a Significant Induction of Apoptosis of LNCaP Cells

Our next goal was to assess whether or not the observed antiproliferative effects of resveratrol against prostate cancer cells are mediated via apoptosis. As shown by terminal nucleotidyl transferase–mediated nick end labeling assay, we found that resveratrol causes a concentration-dependent increase in the apoptosis of LNCaP cells;

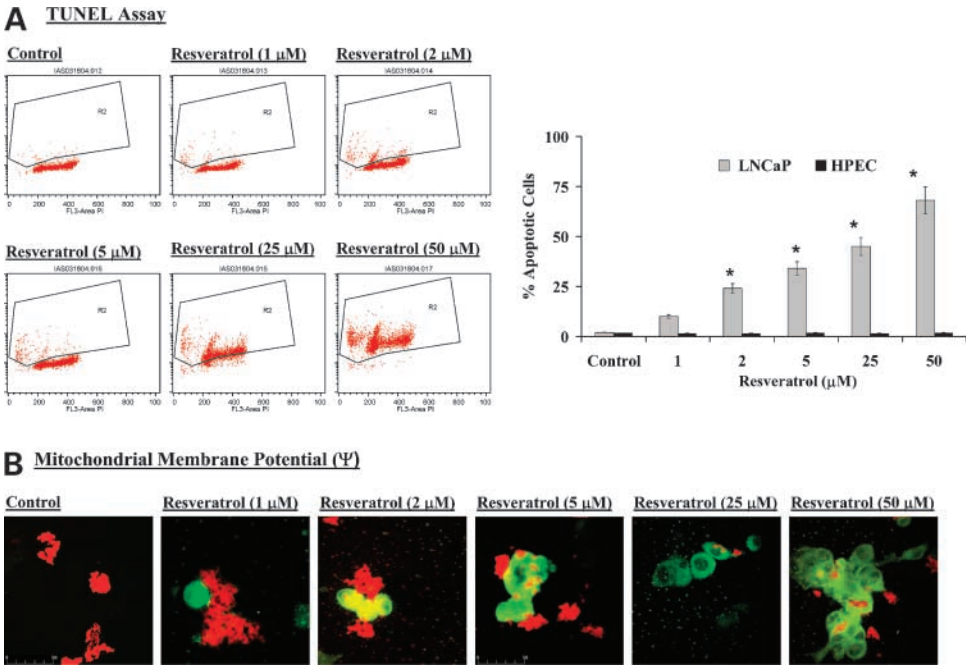


**Figure 2.** Effect of resveratrol on apoptosis in LNCaP and HPEC cells. **A,** terminal nucleotidyl transferase–mediated nick end labeling assay. The growing cells were treated with resveratrol (0–50 μmol/L) for 24 h and the extent of apoptosis was assessed by using APO-Direct assay kit (Phoenix Flow Systems). The analyses were done using Cell Quest software (BD Biosciences); *columns,* mean of three experiments; *bars,* ±SE (*, $P < 0.01$). **B,** mitochondrial membrane potential. For this assay, the growing cells were treated with resveratrol (0–50 μmol/L) or vehicle (DMSO) for 24 h. Cells were labeled with JC-1 dye and analyzed by fluorescence microscopy as per the manufacturer's protocol. In live nonapoptotic cells, the mitochondria appear red following aggregation of APO LOGIC JC-1 reagent. In apoptotic cells, the dye remains in its monomeric form and appears green. Results are from a representative experiment repeated thrice with similar results. Details of the experiments are given in Materials and Methods.



Downloaded from mct.aacrjournals.org on July 27, 2017. © 2006 American Association for Cancer Research.

whereas, interestingly, the normal HPEC cells did not show an induction of apoptosis even at the highest observed concentration of resveratrol when compared with control cells (Fig. 2).

Furthermore, to verify our data on the induction of apoptosis by resveratrol, we measured its effect on the mitochondrial membrane potential ($\Psi$) using a fluorescent cationic dye, known as JC-1. This technique effectively detects apoptosis even at very early stages. In healthy (nonapoptotic) cells, the mitochondria appear red following aggregation of APO LOGIC JC-1 reagent. In apoptotic and dead cells, the dye remains in its monomeric form and appears green. Thus, as shown in Fig. 2B, resveratrol treatment of LNCaP cells resulted in concentration-dependent loss in mitochondrial membrane potential, as compared with control. Similar to the findings of terminal nucleotidyl transferase–mediated nick end labeling assays (Fig. 2A and B), we did not find any effect of resveratrol on the mitochondrial membrane potential in HPEC cells (data not shown).

### The Antiproliferative Response of Resveratrol for LNCaP Cells Was Mediated via Modulations in the PI3K/Akt Pathway

We next investigated whether or not PI3K/Akt was involved in resveratrol-mediated apoptosis of LNCaP cells. As shown by immunoblot analysis and its densitometric quantitation (Fig. 3), resveratrol treatment resulted in an appreciable down-regulation in the protein levels of PI3K/p110, PI3K/p85, and phospho-Ser$^{473}$-Akt (Fig. 3) without an effect on whole Akt in LNCaP cells. These observations suggested that the PI3K/Akt pathway could be involved in the antiproliferative/proapoptotic response of resveratrol against prostate cancer cells.

### Resveratrol Treatment Resulted in a Modulation in the Levels of Bcl-2 Family Proteins in a Fashion that Promotes Apoptosis in LNCaP Cells

Studies have suggested that Bcl-2 could be a crucial mediator downstream of PI3K/Akt signaling, whereas Akt has been shown to negatively regulate the activity of proapoptotic members of the Bcl-2 family (25–27). Therefore, we examined the effect of resveratrol treatment on Bcl-2 family proteins in LNCaP cells. As shown by immunoblot analysis and its densitometric quantitation (Fig. 4), resveratrol treatment of LNCaP cell lines resulted in decrease in antiapoptotic Bcl-2 and a concomitant increase in proapoptotic Bax proteins, thereby causing a significant increase in the Bax/Bcl-2 ratio that favors apoptosis. We also assessed the involvement of other Bcl-2 family proteins (Bad, Bid, and Bax) during the resveratrol-mediated apoptotic death of LNCaP cells. Our data clearly shows that resveratrol causes an increase in the protein levels of proapoptotic members of the Bcl-2 family, i.e., Bid, Bad, and Bak in LNCaP cells (Fig. 5).

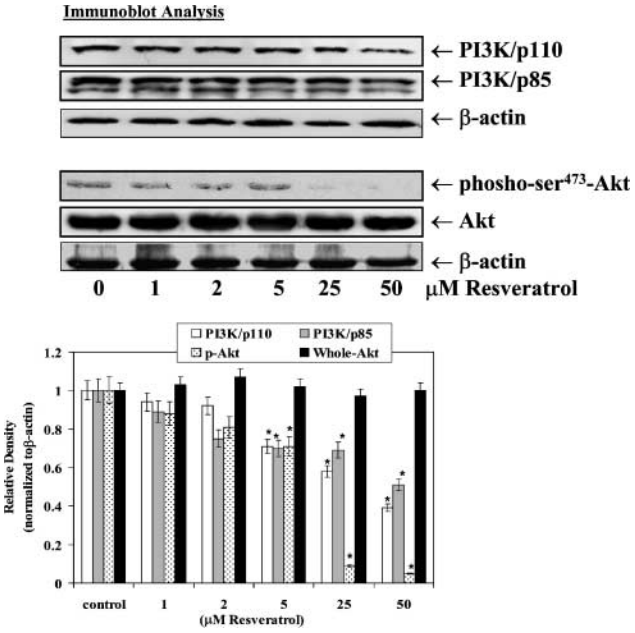


**Figure 3.** Effect of resveratrol on the levels of PI3K/p110, PI3K/p85, phospho-Akt, and whole Akt proteins in LNCaP cells. The cells were treated with resveratrol (0–50 μmol/L) for 24 h, harvested, and the levels of specific proteins were assessed by immunoblot analysis. Equal loading was confirmed by stripping the blot and reprobing it for β-actin. The bands shown here are from a representative experiment repeated thrice with similar results. *Columns,* mean of three experiments; *bars,* ±SE (*, $P < 0.01$). Details of the experiments are given in Materials and Methods.

## Discussion

Resveratrol (*trans*-3,4′,5,-trihydroxystilbene), a phytoalexin found in grapes, nuts, fruits, and red wine, is a potent antioxidant with antiinflammatory, cancer chemopreventive and therapeutic properties (2–9, 11–15). This study was conducted to evaluate the chemopreventive/therapeutic potential of resveratrol against prostate cancer and its mechanism of action. Consistent with earlier observations (12–15), in this study, we found that resveratrol inhibits the proliferation (malignant transformation) and viability of human prostate cancer LNCaP cells via an induction of apoptosis. However, an important observation of our investigation is that resveratrol did not cause apoptosis or result in a decreased viability of normal HPEC cells (isolated from normal prostate tissue obtained during cystoprostatectomy of patients with prostate cancer), even at the highest tested concentration of resveratrol (50 μmol/L). This is important because an ideal chemopreventive or therapeutic drug should be able to eliminate the cancer cells without any toxicity to the normal cells.

Studies have suggested several mechanisms for resveratrol-mediated apoptosis of cancer cells (2). For example, recent studies have shown that resveratrol induces DNA damage in many human cancer cell lines (31, 32) and that it is capable of binding to DNA and cleave or damage DNA in a $Cu^{2+}$-dependent pathway (33). Another recent study has shown that resveratrol causes Cdc2-tyr15 phosphorylation via the ATM/ATR-Chk1/2-Cdc25C pathway leading to DNA damage and S phase arrest selectively in ovarian cancer cells (34).



Downloaded from mct.aacrjournals.org on July 27, 2017. © 2006 American Association for Cancer Research.

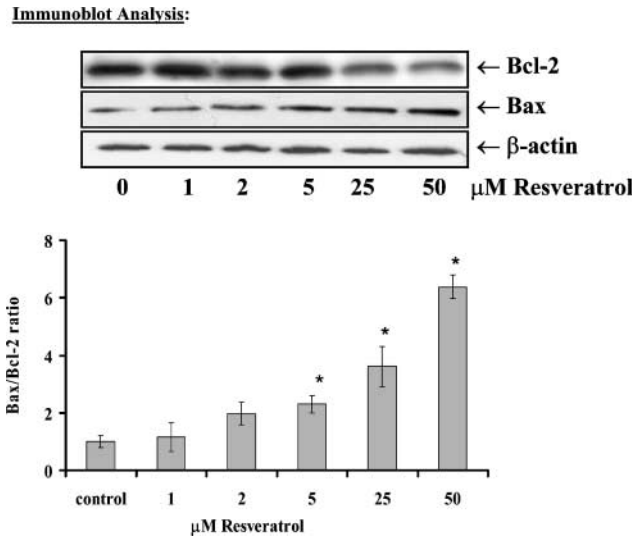


**Figure 4.** Effect of resveratrol on the levels of Bax and Bcl-2 proteins in LNCaP cells. The cells were treated with resveratrol (0 – 50 μmol/L) for 24 h, harvested and the levels of specific proteins were assessed by immunoblot analysis. Equal loading was confirmed by stripping the blot and reprobing it for β-actin. Bands are from a representative experiment repeated thrice with similar results. *Columns*, mean of three experiments (relative density normalized to β-actin); *bars*, ±SE (*, $P < 0.01$). Details of the experiments are given in Materials and Methods.

Here, we studied the involvement of the PI3K/Akt pathway, which has been shown to play a critical role in the development of several human cancers (16–24) in the antiproliferative effects of resveratrol. Studies have suggested that the PI3K/Akt pathway is constitutively active in prostate cancer and represents a major target to prevent dysfunctions in cell growth, survival, and motility (16–24). Our data showed that resveratrol treatment resulted in significant dose-dependent inhibition in the constitutively elevated levels of PI3K/p110 and PI3K/p85 as well as the phosphorylated (active) Akt (at $Ser^{473}$) in LNCaP cells. Akt is an established downstream effector of PI3K and has often been implicated in prostate cancer (23, 35–37). Studies in prostate cancer cell lines have revealed that Akt activation is probably important for the progression of prostate cancer to an androgen-independent state (24). Investigations of human prostate cancer tissues have shown that that although there is neither Akt gene amplification nor enhanced protein expression in prostate cancer compared with normal tissue, poorly differentiated tumors exhibit increased expression of a phosphorylated (activated) form of Akt compared with normal tissue, prostatic intraepithelial neoplasia, or well-differentiated prostate cancer (23). For these reasons, Akt is being investigated as a rational target for drug development for the management of prostate cancer (38). Our study has clearly shown that resveratrol down-modulates the activation (phosphorylation) of Akt and, therefore, could be useful for the prevention as well as therapy of prostate cancer.

Because the activation of the PI3K/Akt pathway leads to increased expression of Bcl-2 (26, 27), we assessed the effect of resveratrol on Bcl-2 family proteins; and our data showed that resveratrol treatment of LNCaP cells (*a*) down-regulates Bcl-2 protein, and (*b*) up-regulates the protein levels of proapoptotic members of this family, i.e., Bax, Bid, Bad, and Bak. We also found that resveratrol resulted in a significant down-modulation of the Bax/Bcl-2 ratio that is regarded as a driving force for apoptosis in mammalian cells. Furthermore, our data also showed a loss of mitochondrial membrane potential in resveratrol-treated LNCaP cells, suggesting that inhibition of Akt activation is possibly preceded by modulations in mitochondrial damage leading to a shift in the balance between proapoptotic and antiapoptotic proteins in favor of apoptosis.

Taken together, our data suggested that resveratrol imparts significant antiproliferative response against human prostate cancer cells without any appreciable effect in normal prostate cells and PI3K/Akt pathway plays an important role in the chemopreventive/antiproliferative effects of this polyphenolic antioxidant. This is important because the PI3K/Akt pathway was shown to be constitutively activated in human prostate cancer. Based on the outcome of this study, we suggest that resveratrol could be developed as an agent for the management of prostate cancer. However, further studies are needed to establish a cause-and-effect relationship between PI3K/Akt pathway and resveratrol effect. Furthermore, studies are also needed to define the upstream signaling that is involved in the inhibition of the PI3K/Akt pathway by resveratrol in

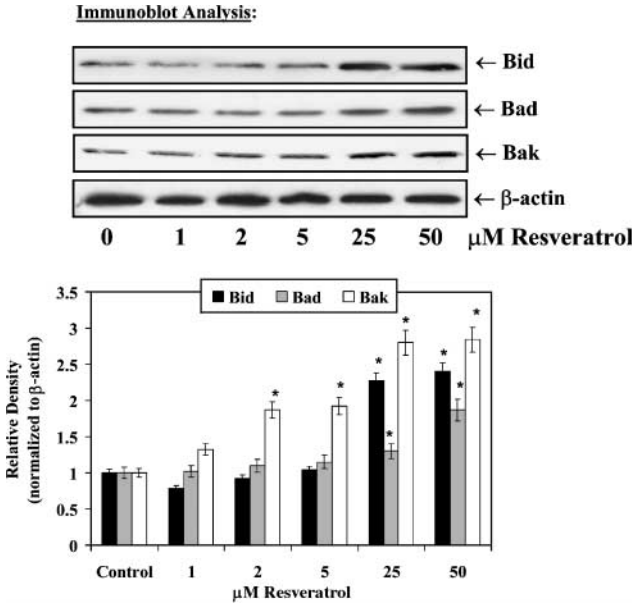


**Figure 5.** Effect of resveratrol on the levels of Bid, Bad, Bak, and survivin proteins in LNCaP cells. The cells were treated with resveratrol (0 – 50 μmol/L) for 24 h, harvested, and the levels of specific proteins were assessed by immunoblot analysis. Equal loading was confirmed by stripping the blot and reprobing it for β-actin. Bands from a representative experiment repeated thrice with similar results. *Columns*, mean of three experiments (relative density normalized to β-actin); *bars*, ±SE (*, $P < 0.01$). Details of the experiments are given in Materials and Methods.



Downloaded from mct.aacrjournals.org on July 27, 2017. © 2006 American Association for Cancer Research.

human prostate cancer cells. The involvement of PTEN is an intriguing possibility in this direction because the mutations in this gene lead to constitutive activation of Akt with phosphorylation at the protein kinase B/Akt sites $Thr^{308}$ and $Ser^{473}$. Furthermore, it will also be important to validate these findings in *in vivo* animal models having relevance to human situations.

### References


**1.** Jemal A, Murray T, Ward E, et al. Cancer statistics, 2006. CA Cancer J Clin 2006;56:106 – 30.

**2.** Aziz MH, Kumar R, Ahmad N. Cancer chemoprevention by resveratrol: *in vitro* and *in vivo* studies and the underlying mechanisms (review). Int J Oncol 2003;23:17 – 28.

**3.** Gupta S, Ahmad N, Mukhtar H. Prostate cancer chemoprevention by green tea. Semin Urol Oncol 1999;17:70 – 6.

**4.** Bianchini F, Vainio H. Wine and resveratrol: mechanisms of cancer prevention? Eur J Cancer Prev 2003;12:417 – 25.

**5.** Savouret JF, Quesne M. Resveratrol and cancer: a review. Biomed Pharmacother 2002;56:84 – 7.

**6.** Aggarwal BB, Bhardwaj A, Aggarwal RS, et al. Role of resveratrol in prevention and therapy of cancer: preclinical and clinical studies. Anticancer Res 2004;24:2783 – 840.

**7.** Bhat KP, Pezzuto JM. Cancer chemopreventive activity of resveratrol. Ann N Y Acad Sci 2002;957:210 – 29.

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

**9.** Jang M, Cai L, Udeani GO, et al. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. Science 1997;275: 218 – 20.

**10.** Goldberg DM, Hahn SE, Parkes JG. Beyond alcohol: beverage consumption and cardiovascular mortality. Clin Chim Acta 1995;237: 155 – 87.

**11.** Jones SB, DePrimo SE, Whitfield ML, et al. Resveratrol-induced gene expression profiles in human prostate cancer cells. Cancer Epidemiol Biomarkers Prev 2005;14:596 – 604.

**12.** Kim YA, Rhee SH, Park KY, et al. Antiproliferative effect of resveratrol in human prostate carcinoma cells. J Med Food 2003;6:273 – 80.

**13.** Narayanan BA, Narayanan NK, Re GG, et al. Differential expression of genes induced by resveratrol in LNCaP cells: P53-mediated molecular targets. Int J Cancer 2003;104:204 – 12.

**14.** Kampa M, Hatzoglou A, Notas G, et al. Wine antioxidant polyphenols inhibit the proliferation of human prostate cancer cell lines. Nutr Cancer 2000;37:223 – 33.

**15.** Hsieh TC, Wu JM. Differential effects on growth, cell cycle arrest, and induction of apoptosis by resveratrol in human prostate cancer cell lines. Exp Cell Res 1999;249:109 – 15.

**16.** Osaki M, Oshimura M, Ito H. PI3K-Akt pathway: its functions and alterations in human cancer. Apoptosis 2004;9:667 – 76.

**17.** Wetzker R, Rommel C. Phosphoinositide 3-kinases as targets for therapeutic intervention. Curr Pharm Des 2004;10:1915 – 22.

**18.** Fresno Vara JA, Casado E, de CJ, et al. PI3K/Akt signalling pathway and cancer. Cancer Treat Rev 2004;30:193 – 204.

**19.** Franke TF, Hornik CP, Segev L, et al. PI3K/Akt and apoptosis: size matters. Oncogene 2003;22:8983 – 98.

**20.** Luo J, Manning BD, Cantley LC. Targeting the PI3K-Akt pathway in human cancer: rationale and promise. Cancer Cell 2003;4:257 – 62.

**21.** Sharma M, Chuang WW, Sun Z. Phosphatidylinositol 3-kinase/Akt stimulates androgen pathway through GSK3$\beta$ inhibition and nuclear $\beta$-catenin accumulation. J Biol Chem 2002;277:30935 – 41.

**22.** Pfeil K, Eder IE, Putz T, et al. Long-term androgen-ablation causes increased resistance to PI3K/Akt pathway inhibition in prostate cancer cells. Prostate 2004;58:259 – 68.

**23.** Ghosh PM, Malik S, Bedolla R, et al. Akt in prostate cancer: possible role in androgen-independence. Curr Drug Metab 2003;4:487 – 96.

**24.** Murillo H, Huang H, Schmidt LJ, et al. Role of PI3K signaling in survival and progression of LNCaP prostate cancer cells to the androgen refractory state. Endocrinology 2001;142:4795 – 805.

**25.** Kumar P, Miller AI, Polverini PJ. p38 MAPK mediates $\gamma$-irradiation-induced endothelial cell apoptosis, and vascular endothelial growth factor protects endothelial cells through the phosphoinositide 3-kinase-Akt-Bcl-2 pathway. J Biol Chem 2004;279:43352 – 60.

**26.** Asnaghi L, Calastretti A, Bevilacqua A, et al. Bcl-2 phosphorylation and apoptosis activated by damaged microtubules require mTOR and are regulated by Akt. Oncogene 2004;23:5781 – 91.

**27.** Jin YP, Fishbein MC, Said JW, et al. Anti-HLA class I antibody-mediated activation of the PI3K/Akt signaling pathway and induction of Bcl-2 and Bcl-xL expression in endothelial cells. Hum Immunol 2004;65: 291 – 302.

**28.** Schwarze SR, Shi Y, Fu VX, et al. Role of cyclin-dependent kinase inhibitors in the growth arrest at senescence in human prostate epithelial and uroepithelial cells. Oncogene 2001;20:8184 – 92.

**29.** Jarrard DF, Sarkar S, Shi Y, et al. p16/pRb pathway alterations are required for bypassing senescence in human prostate epithelial cells. Cancer Res 1999;59:2957 – 64.

**30.** Garikapaty VP, Ashok BT, Tadi K, et al. Synthetic dimer of indole-3-carbinol: second generation diet derived anti-cancer agent in hormone sensitive prostate cancer. Prostate 2006;66:453 – 62.

**31.** Sgambato A, Ardito R, Faraglia B, et al. Resveratrol, a natural phenolic compound, inhibits cell proliferation and prevents oxidative DNA damage. Mutat Res 2001;496:171 – 80.

**32.** Bove K, Lincoln DW, Tsan MF. Effect of resveratrol on growth of 4T1 breast cancer cells *in vitro* and *in vivo*. Biochem Biophys Res Commun 2002;291:1001 – 5.

**33.** Fukuhara K, Nagakawa M, Nakanishi I, et al. Structural basis for DNA-cleaving activity of resveratrol in the presence of Cu(II). Bioorg Med Chem 2006;14:1437 – 43.

**34.** Tyagi A, Singh RP, Agarwal C, et al. Resveratrol causes Cdc2 – 15 phosphorylation via ATM/ATR-Chk1/2-Cdc25C pathway as a central mechanism for S phase arrest in human ovarian carcinoma Ovcar-3 cells. Carcinogenesis 2005;26:1978 – 87.

**35.** Li L, Ittmann MM, Ayala G, et al. The emerging role of the PI3-K-Akt pathway in prostate cancer progression. Prostate Cancer Prostatic Dis 2005;8:108 – 18.

**36.** Shukla S, Maclennan GT, Marengo SR, et al. Constitutive activation of PI3K-Akt and NF-$\kappa$B during prostate cancer progression in autochthonous transgenic mouse model. Prostate 2005;64:224 – 39.

**37.** Kreisberg JI, Malik SN, Prihoda TJ, et al. Phosphorylation of Akt (Ser473) is an excellent predictor of poor clinical outcome in prostate cancer. Cancer Res 2004;64:5232 – 6.

**38.** Pommery N, Henichart JP. Involvement of PI3K/Akt pathway in prostate cancer. Potential strategies for developing targeted therapies. Ann Pharm Fr 2005;63:69 – 75.




Downloaded from mct.aacrjournals.org on July 27, 2017. © 2006 American Association for Cancer Research.

AACR American Association for Cancer Research

Molecular Cancer Therapeutics

# Resveratrol-caused apoptosis of human prostate carcinoma LNCaP cells is mediated via modulation of phosphatidylinositol 3′-kinase/Akt pathway and Bcl-2 family proteins

Moammir H. Aziz, Minakshi Nihal, Vivian X. Fu, et al.

*Mol Cancer Ther* 2006;5:1335-1341.

| | |
|---|---|
| **Updated version** | Access the most recent version of this article at: http://mct.aacrjournals.org/content/5/5/1335 |

| | |
|---|---|
| **Cited articles** | This article cites 38 articles, 7 of which you can access for free at: http://mct.aacrjournals.org/content/5/5/1335.full#ref-list-1 |
| **Citing articles** | This article has been cited by 8 HighWire-hosted articles. Access the articles at: http://mct.aacrjournals.org/content/5/5/1335.full#related-urls |

| | |
|---|---|
| **E-mail alerts** | Sign up to receive free email-alerts related to this article or journal. |
| **Reprints and Subscriptions** | To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org. |
| **Permissions** | To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org. |

Downloaded from mct.aacrjournals.org on July 27, 2017. © 2006 American Association for Cancer Research.